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A Shared Genetic Basis for Self-Limited Delayed Puberty and Idiopathic Hypogonadotropic Hypogonadism

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TABLE OF CONTENTS:

ABSTRACT: [*]

Background: Delayed puberty is a common condition and, in the absence of an underlying condition, is self-limited in most cases. Though delayed puberty appears to be heritable, no specific genetic cause has yet been reported. In contrast, many genetic causes have been found for idiopathic hypogonadotropic hypogonadism (IHH), a rare disorder in which defects in GnRH secretion or action lead to absent or stalled pubertal development. We hypothesized that there is a shared genetic basis for both self-limited delayed puberty and IHH.

Methods: We used two approaches to determine if there is genetic overlap between self-limited delayed puberty and IHH. First, in pedigrees with a proband with IHH known to carry a variant in an IHH gene, we performed targeted sequencing to determine whether family members with self-limited delayed puberty were more likely than family members with normal pubertal timing to share the proband's variant. Second, in probands with self-limited delayed puberty and no family history of IHH, we performed whole-exome sequencing and examined 33,855 ethnically matched controls drawn from the Exome Aggregation Consortium for variants in 21 IHH genes. Variants were characterized as potentially pathogenic based on rarity, severity of mutation, and *in silico* analyses.

Results: In pedigrees with an IHH proband, the proband's potentially pathogenic variant was shared by 53% (10/19) of delayed puberty family members vs. 12% (4/33) of unaffected family members ($P = 0.003$). In delayed puberty subjects with no family history of IHH, 14% (8/56) had potentially pathogenic variants in IHH genes vs. 5.6% (1,907/33,855) of controls ($P = 0.01$). Such variants were found at a higher frequency in subjects with self-limited delayed puberty compared to controls in the specific genes *IL17RD*, *TAC3*, and *TACR3*.

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Conclusions: For the first time, we report a specific genetic cause for self-limited delayed puberty. These findings suggest that variants in IHH genes can contribute to the pathogenesis of self-limited delayed puberty. Thus, at least in some cases, self-limited delayed puberty shares an underlying pathophysiology with IHH. In addition, our study presents *IL17RD*, *TAC3*, and *TACR3* as candidate genes for future genetic studies of self-limited delayed puberty and highlights both the benefits and limitations of genetic testing.

GLOSSARY OF ABBREVIATIONS AND GENE NAMES:

Note: All human gene names are italicized, and the names of proteins encoded by the corresponding gene are provided in the right column.

ALS	Acid-labile subunit
APOE	Apolipoprotein E
AVPV/PeN	Anteroventral periventricular nucleus/rostral periventricular nucleus
BCH	Boston Children's Hospital
CDGP	Constitutional delay of growth and puberty
CHD7	Chromodomain helicase DNA-binding protein 7
DMXL2	Rabconnectin-3
DUSP6	Dual specificity phosphatase 6
DYN	Dynorphin A
ExAC	Exome Aggregation Consortium
FEZF1	FEZ family zinc finger 1
FGF8	Fibroblast growth factor 8
FGF17	Fibroblast growth factor 17
FGFR1	Fibroblast growth factor receptor 1
FLRT3	Fibronectin leucine rich transmembrane protein 3
FSH	Follicle-stimulating hormone
GH	Growth hormone
GHSR	Growth hormone secretagogue receptor
GnRH	Gonadotropin-releasing hormone

GNRH1	Gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)
GNRHR	Gonadotropin-releasing hormone receptor
HESX1	Homeobox gene expressed in ES cells 1
HS6ST1	Heparan sulfate 6-O-sulfotransferase 1
IGFALS	Insulin-like growth factor-binding protein, acid-labile subunit
IGSF1	Immunoglobulin superfamily, member 1
IHH	Idiopathic hypogonadotropic hypogonadism
IL17RD	Interleukin 17 receptor D
KAL1	Anosmin-1
KCNJ11	Potassium inwardly-rectifying channel, subfamily J, member 11
KISS1	KiSS-1 metastasis-suppressor
KISS1R	KISS1 receptor
KNDy	Kisspeptin/neurokinin B/dynorphin
LEP	Leptin
LEPR	Leptin receptor
LH	Luteinizing hormone
LIN28B	Protein lin-28 homolog B
NR0B1 (DAX1)	Nuclear receptor subfamily 0, group B, member 1 (Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1)
NR5A1 (SF1)	Nuclear receptor subfamily 5, group A, member 1 (Steroidogenic factor 1)

NSMF (NELF)	NMDA receptor synaptonuclear signaling and neuronal migration factor (Nasal embryonic luteinizing hormone-releasing hormone factor)
NHLBI GO ESP	National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project
NKB	Neurokinin B
MGH REU	Massachusetts General Hospital Reproductive Endocrine Unit
OTUD4	OUT domain-containing protein 4
PCSK1	Proprotein convertase, subtilisin/kexin-type 1
PNPLA6	Patatin-like phospholipase domain-containing protein 6
POLR3A	Polymerase III, RNA, subunit A
POLR3B	Polymerase III, RNA, subunit B
PROK2	Prokineticin 2
PROKR2	Prokineticin receptor 2
RB&C	Rainbow Babies and Children's Hospital
RNF216	Ring finger protein 216
SEMA3A	Semaphorin-3A
SOX10	SRY-Box 10
SPRY4	Sprouty homolog 4 (Drosophila)
STUB1	STIP1 homologous and U box-containing protein 1
TAC3	Tachykinin 3 (also called neurokinin B)
TACR3	Tachykinin receptor 3
TUBB3	Tubulin, beta-3
WDR11	WD repeat domain 11

1 INTRODUCTION:

Puberty is a striking period of development that bridges childhood and adulthood. It is characterized by rapid growth and development of sexual maturity and fertility. Though puberty has intrigued scientists for decades, the factors that initiate puberty and regulate pubertal timing remain elusive.[1] Human disease models in which pubertal timing is altered offer a unique opportunity to identify these still unknown factors.

1.1 Function of the hypothalamic-pituitary-gonadal (HPG) axis in puberty

Pubertal development is governed by the hypothalamic-pituitary-gonadal (HPG) axis. At the onset of puberty, neurons in the hypothalamus release gonadotropin-releasing hormone (GnRH), which in turn binds to its receptors in the anterior pituitary to stimulate the production and release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In early puberty, LH and FSH pulses first appear during sleep and gradually increase in both frequency and amplitude during pubertal development and eventually become detectable during daytime.[2, 3] The gonadotropins LH and FSH subsequently stimulate the gonads to secrete sex steroids, which initiate the development of secondary sexual characteristics. In boys, the first physical sign that indicates the onset of puberty is testicular growth to a volume of 4 mL or greater. In girls, the first sign is the development of breast tissue or thelarche, which is followed by the onset of menstruation or menarche approximately 2 years later.[4, 5] Sex steroids and increasing growth hormone (GH) levels initiate a period of rapid skeletal growth often referred to as the pubertal growth spurt.[6] Following puberty, ongoing GnRH secretion is essential for the maintenance of secondary sexual characteristics, sexual function, and fertility throughout adulthood.

1.2 Regulation of the HPG axis

A complex network of regulatory pathways governs the awakening of the HPG axis at puberty. This network synthesizes various hormonal, psychological, and environmental cues, which together determine pubertal onset.[1] While the influences on pubertal timing, including nutritional status, systemic illness, and psychological stress, are known, the exact molecular mechanisms that "kick-start" GnRH secretion are still a mystery.[1]

Recent work has identified a novel neuron, the kisspeptin neuron in the hypothalamus, as a key regulator of GnRH secretion and, thus, pubertal timing.[7] The kisspeptin neuron functions upstream of the GnRH neuronal network to release the peptide kisspeptin, which binds to its receptor located on GnRH neurons and subsequently stimulates the pulsatile release of GnRH that drives the activation of the HPG axis.[8, 9] Kisspeptin has since been shown to play key roles in both positive and negative feedback of sex steroids on the GnRH neuronal network in rodent models with kisspeptin-secreting neurons in different locations mediating different effects.[10] Kisspeptin neurons in the arcuate nucleus (ARC) are involved in negative feedback, and studies in rodents show that kisspeptin neurons in the anteroventral periventricular nucleus/rostral periventricular nucleus (AVPV/PeN) have a positive feedback role thought to mediate the LH surge necessary for ovulation (Figure 1).[7]

Further work on the regulation of kisspeptin signaling has revealed two additional neurotransmitters that are coexpressed with kisspeptin in the arcuate nucleus within the hypothalamus, neurokinin B (NKB) and dynorphin A (DYN). These kisspeptin-secreting neurons are known collectively as KNDy neurons, named for their coexpression of Kisspeptin/Neurokinin B/Dynorphin. KNDy neurons have been shown to function as a complex interconnected network thought to regulate GnRH secretion through both positive (NKB) and negative (DYN) modulation of kisspeptin signaling (Figure 1).[10]

1.3 Origin of the GnRH neuronal network

Animal studies have shown that GnRH neurons originate predominantly from the olfactory placodes,[11] ectodermal tissue that ultimately differentiates to form the olfactory epithelium.[12] Following differentiation, the GnRH neurons leave the olfactory placode and migrate to their final destination in the hypothalamus with the guidance of nerve fibers from the olfactory neurons. Once they reach the hypothalamus, the GnRH neurons project their axons to the median eminence, where they access the hypophyseal portal system that delivers hormones to the pituitary.[11] Though little is known about what molecules and factors guide GnRH neurons to their final destination in the hypothalamus, cues from secreted extracellular molecules, growth factors, chemokines, neurotransmitters, and ion channels are all thought to play a role.[11]

1.4 Clinical features and evaluation of delayed puberty

Delayed puberty is commonly defined as the lack of sexual maturation at an age greater than 2 or more standard deviations above the mean for a given population.[13] In conventional clinical practice, this leads to evaluation of boys who have not achieved a testicular volume of 4 mL or greater by 14 years and girls who have not started breast development by 13 years.[4, 5, 14-17] However, a recent downward trend in pubertal timing has been observed in the U.S[18-21] and other developed countries[22-25] with significant variability between racial and ethnic groups, which is not yet reflected in the clinical age conventions. Thus, these traditional age cut-offs must be interpreted with caution, as they may not accurately represent the normative spectrum of puberty timing for current populations.

Several conditions can present with delayed puberty, and the differential diagnoses can be divided into four main categories: 1) hypergonadotropic hypogonadism or primary gonadal

insufficiency, 2) functional hypogonadotropic hypogonadism, 3) permanent hypogonadotropic hypogonadism, and 4) self-limited delayed puberty.[13]

1.4.1 HYPERGONADOTROPIC HYPOGONADISM

Hypergonadotropic hypogonadism, also known as primary gonadal insufficiency, is caused by either a congenital or acquired primary defect of the gonads and accounts for approximately 10-20% of all delayed puberty presentations.[26] Common etiologies include Turner's syndrome, a genetic syndrome in which monosomy of the X chromosome results in ovarian dysfunction, and acquired defects in the gonads secondary to chemotherapy or radiation.[13] The absence of negative sex-steroid feedback from the gonads in these disorders results in elevated levels of the gonadotropins LH and FSH and thus, hypergonadotropic hypogonadism.

1.4.2 FUNCTIONAL HYPOGONADOTROPIC HYPOGONADISM

Functional hypogonadotropic hypogonadism, a condition in which an underlying disorder delays maturation of the HPG axis, is the underlying etiology of delayed puberty in approximately 20% of all delayed-puberty cases.[26] In girls, this is often referred to as functional hypothalamic amenorrhea. This is predominantly a transient condition in which stress from a systemic illness (inflammatory bowel disease or anorexia nervosa), environmental stressor (excessive exercise), or other endocrinopathy (hypothyroidism) essentially "shuts down" the HPG axis. This central suppression results in low gonadotropins levels and thus, delayed maturation of the gonads, which is reversible with treatment of the underlying condition.[13]

1.4.3 PERMANENT HYPOGONADOTROPIC HYPOGONADISM

In approximately 13% of cases, hypogonadotropic hypogonadism can be a permanent disorder, which can be caused by anatomic defects in the central nervous system such as a tumor, congenital syndromes such as hypopituitarism or Prader-Willi syndrome, or inflammatory

conditions such as hypophysitis.[26] In cases when no underlying etiology for permanent hypogondatropic hypogonadism can be identified, a diagnosis of congenital or idiopathic hypogonadotropic hypogonadism (IHH) is made. In IHH, defects in the secretion or action of GnRH lead to low levels of sex-steroids in the presence of inappropriately low levels of gonadotropins. Clinically, individuals with IHH have absent or stalled pubertal development by adult age (commonly defined as 18 years). Additional clinical features and the genetic etiology of IHH will be further discussed in Section 1.5 Idiopathic hypogonadotropic hypogonadism (IHH).

1.4.4 SELF-LIMITED DELAYED PUBERTY

The most common cause of delayed puberty in both boys and girls is self-limited delayed puberty, in which individuals progress through puberty spontaneously prior to reaching adult age (18 years). Over 50% of all delayed-puberty cases are due to self-limited delayed puberty.[26] A subset of individuals with self-limited delayed puberty also have delayed longitudinal growth pattern, an entity known as constitutional delay of growth and puberty (CDGP).[26] These diagnoses can only be made after all other conditions have been ruled out, and spontaneous initiation and progression of puberty occurs after a period of clinical observation.

1.4.5 DIAGNOSTIC DILEMMAS

Prior to age 18 years, it can be difficult to clinically distinguish patients with self-limited delayed puberty from those with IHH, as both conditions are diagnoses of exclusion. While self-limited delayed puberty resolves on its own, most cases of IHH require lifelong hormone therapy.[13, 27] Several laboratory tests have been suggested to differentiate the two disorders. Basal gonadotropin levels in the pubertal range can occur prior to the physical signs of puberty, and, thus, suggest a diagnosis of self-limited delayed puberty. If basal gonadotropins are low, successful stimulation of the HPG axis by GnRH or a GnRH agonist can also suggest a diagnosis

of self-limited delayed puberty, as stimulated LH levels in the pubertal range can indicate HPG axis activation and imminent pubertal initiation.[28] However, the lack of a response to GnRH stimulation cannot differentiate between the two disorders, as the HPG axis may not yet be active in an individual with self-limited delayed puberty and may be permanently defective in an individual with IHH.[13] To date, no test is available to definitively distinguish between these two patient populations. In the absence of a reliable prognostic test, expectant observation remains a standard approach in a child presenting with delayed puberty after underlying conditions are ruled out, which can affect psychosocial well-being and delay definitive treatment.[13]

Understanding the genetics of self-limited delayed puberty is a critical step to determining the factors that regulate pubertal timing, which has the potential to guide the development of new and effective diagnostic tools and therapies for delayed puberty and other reproductive endocrine disorders.

1.5 Idiopathic hypogonadotropic hypogonadism (IHH)

1.5.1 CLINICAL FEATURES

IHH is commonly defined as the lack of sexual maturation by age 18 years, with low sex steroids and inappropriately low/normal gonadotropins in the absence of any other identifiable causes of hypogonadotropic hypogonadism, such as systemic illness, anatomical defect, or other endocrinopathy as discussed in Section 1.4.3. Permanent hypogonadotropic hypogonadism.[27] Congenital IHH has been traditionally classified into two entities based on olfactory phenotype. IHH with a normal sense of smell is known as normosmic IHH and with defective or absent sense of smell as Kallmann's syndrome.[1] Over 60% of individuals with congenital IHH have defective or absent smell.[29] The defect in olfaction in Kallmann's syndrome has been suggested to result from defects in the migration of olfactory neurons, which in turn disrupts the migration

of GnRH neurons.[30] In contrast, normosmic IHH is thought to result from defects in the action or secretion of GnRH.[31]

The reproductive phenotypic spectrum of IHH is broad, ranging from absent pubertal development and undetectable serum gonadotropins to partial pubertal development with spermatogenesis in men and spontaneous thelarche in women.[32, 33] In the majority of cases, individuals with IHH require life-long sex steroid replacement and fertility induction with pulsatile GnRH or exogenous gonadotropins.[13] Notably, up to 20% of individuals with IHH undergo "reversal" of their condition with activation of the hypothalamic-pituitary-gonadal axis and normalization of reproductive endocrine function in adulthood.[34-42] Though the exact factors that mediate reversal are unclear, exposure to sex steroids has been suggested to enhance the late activation of the GnRH neuronal network.[39]

1.5.2 GENETICS OF IHH

Over the past 20 years, significant advances have been made in understanding the genetics underlying IHH (whether in isolation or in the context of a broader syndrome), with over thirty genes now implicated in the development, migration, and secretory function of GnRH neurons.[1, 43-52] These genes have been broadly classified as neurodevelopmental genes critical to the development and migration of GnRH neurons, or neuroendocrine genes critical to the regulation of GnRH secretion at the level of the hypothalamus or action at the level of pituitary (Figure 2).[1]

The majority of genes associated with isolated IHH have been implicated in the development and migration of GnRH neurons. Notable examples include *FGF8* and *FGFR1*, which encode the ligand fibroblast growth factor 8 and its receptor fibroblast growth factor receptor 1. These genes are involved in the FGF signaling pathway, a system that plays critical roles in mitogenesis, tissue differentiation, and cell migration and survival.[53] Studies in mice

show that both *FGF8* and *FGFR1* play critical roles in development of GnRH neurons.[53] The first variants in *FGFR1* and *FGF8* were first described in humans with IHH in 2003[54] and 2008,[55] respectively. Two additional genes implicated in IHH, *KAL1* and *HS6ST1*, are believed to be modulators of FGF signaling.[56] Furthermore, recent investigations of a group of genes with similar roles in neuronal development, referred to as the "FGF8 synexpression group," have implicated an additional six genes (*FGF17*, *IL17RD*, *DUSP6*, *SPRY4*, and *FLRT3*) in the pathogenesis of IHH.[44]

Several genes have been shown to play a role in the regulation of GnRH secretion or action. *GNRH1*, the gene that encodes the prohormone that is ultimately processed to GnRH, and *GNRHR*, which encodes the receptor for GnRH, were obvious candidates, and variants in both have been identified in individuals with IHH.[57, 58] Variants in the upstream regulators of GnRH secretion, kisspeptin and its receptor, encoded by the genes *KISS1* and *KISS1R*, respectively, have also been identified in individuals with IHH.[8, 9, 59] Most recently, the neurokinin B pathway, which is believed to positively regulate kisspeptin signaling as part of the KNDy neuronal system in the arcuate nucleus of the hypothalamus, has been implicated in the pathogenesis of IHH.[10] Variants in both *TAC3* and *TACR3*, genes that encode neurokinin B and its receptor, respectively, have been identified in individuals with IHH.[60] Of note, IHH with reversal, a subtype of IHH in which individuals spontaneously undergo puberty after adulthood, has specifically been associated with rare variants in *TAC3* and *TACR3*.[34, 40]

IHH can also be observed as one feature in a broader syndrome. Examples include Gordon Holmes syndrome, a rare neurological disorder characterized by ataxia, dementia, and hypogonadism, which has been recently associated with variants in the ubiquitination proteins encoded by *RNF216* and *OTUD4*,[45] and CHARGE syndrome (an acronym for the multiple congenital anomalies associated with the syndrome: coloboma, heart anomalies, choanal atresia,

retardation, and genital and ear anomalies), which has been associated with variants in *CHD7*, a transcriptional regulator protein.[61] The role that these syndromic genes play in the development or function of the GnRH neuronal network and the exact reproductive phenotypes associated with variants in these genes are still under investigation (Figure 2).[45-52]

The inheritance patterns of IHH are diverse and include X-linked recessive with mutations in *KAL1*,[62] autosomal dominant with mutations in *FGFR1*,[63] and autosomal recessive with mutations in *KISS1R*[8] and *GNRHR*.[57] Even in genes with established inheritance patterns, the transmission of IHH caused by variants in these genes is not always uniform.[56] For example, a study of IHH individuals with rare variants in *GNRHR* (shown to predominantly follow an autosomal recessive pattern) found that over 40% of familial cases followed an autosomal dominant pattern of inheritance.[64] In addition, variable expressivity and incomplete penetrance have been observed in IHH individuals and their relatives with varying reproductive phenotypes associated with variants in *FGFR1*, *GNRHR*, and *IL17RD*.[32, 44, 65, 66] More recently, some individuals with IHH have been found to have variants in two or more IHH-associated genes, suggesting an oligogenic basis for a condition previously thought to be monogenic.[67] However, the extent to which oligogenic inheritance plays a role in the disorder is still unclear.[68]

1.6 Self-limited delayed puberty

1.6.1 CLINICAL FEATURES

In the absence of underlying medical conditions or anatomic defects, as discussed in Section 1.4 Clinical features and evaluation of delayed puberty, delayed puberty is typically self-limited, with individuals progressing through puberty spontaneously prior to reaching an adult age (18 years).[1] As partial pubertal development with subsequent stalling has been observed in individuals with IHH,[32, 33] self-limited delayed puberty can only be definitively diagnosed after a

period of clinical observation in which the individual initiates and progresses through puberty at a normal rate.[13] A subset of individuals with self-limited delayed puberty also experience longitudinal growth delay, and are further categorized as having CDGP.[26]

1.6.2 UNKNOWN GENETICS OF SELF-LIMITED DELAYED PUBERTY

Self-limited delayed puberty appears to be highly heritable, as 50-80% of these patients have a family history of delayed puberty.[69, 70] Many self-limited delayed puberty pedigrees show an autosomal dominant pattern, suggesting that the condition can be caused by the effects of single-gene mutations.[69, 70]

Given these observations, many candidate genes for self-limited delayed puberty have been proposed. Leptin, a satiety hormone regulates adipose-tissue mass through hypothalamic signaling, and its receptor have previously been implicated in early-onset obesity and delayed puberty.[71] However, polymorphisms in *LEP* and *LEPR,* the genes that encode leptin and its receptor, respectively, have not been found to be associated with isolated self-limited delayed puberty.[72] Another gene implicated in a broader syndrome with delayed puberty is *IGSF1*, which encodes the immunoglobulin superfamily member 1 glycoprotein that is predominately expressed in the pituitary and testes.[73] Though variants in *IGSF1* have been identified in an X-linked syndrome of central hypothyroidism, macroorchidism, and delayed puberty, they are not believed to underlie isolated self-limited delayed puberty.[74]

Genes involved in the growth hormone (GH) signaling pathway have also been explored as candidates in self-limited delayed puberty. The acid-labile subunit gene *IGFALS*, which encodes a growth-hormone dependent peptide involved in protecting insulin-like growth factor-1 from degradation, has been implicated in acid-labile subunit (ALS) deficiency, a disorder characterized by mild growth retardation with pubertal delay.[75] However, no variants in *IGFALS* were identified in a cohort of individuals with self-limited delayed puberty.[76] The GH

segretagogue receptor, *GHSR*, which is stimulated by the gastric hormone ghrelin to secrete GH, has been implicated in idiopathic short stature and partial isolated GH deficiency with delayed puberty.[77] Sequencing in *GHSR* in a group of 96 individuals with idiopathic short stature, including 31 with self-limited delayed puberty, revealed two heterozygous variants with decreased *in vitro* signaling activity in two subjects with self-limited delayed puberty.[78] Though these variants were not identified in controls, future studies with larger cohorts are needed to definitively establish this association.

Large genome-wide linkage and association studies have also been utilized to identify candidate delayed puberty genes and loci associated with variation in normal pubertal timing. In 2008, a genome-wide linkage study revealed a significant association between self-limited delayed puberty and a locus on chromosome 2,[79] but subsequent genome-wide association studies did not detect such a signal. However, four independent genome-wide association studies did identify that variation near or in the gene *LIN28B*, a human homolog of the gene *lin-28* in *C. elegans*, which regulates growth progression, was associated with pubertal timing in humans.[80-83] Two recent meta-analyses confirmed this association and identified over 100 additional loci associated with age at menarche.[84, 85] Furthermore, two studies have also associated variation near or at *LIN28B* with growth in height in addition to pubertal timing.[86, 87] Despite these reports, no variants in *LIN28B* were identified in a cohort of individuals with self-limited delayed puberty.[88]

Given the phenotypic similarities between IHH and self-limited delayed puberty, genes underlying isolated congenital IHH, as discussed in Section 1.5.2 Genetics of IHH, have also been popular candidate genes for delayed puberty. Several studies have investigated the role of both common and rare variants in IHH genes in individuals with self-limited delayed puberty

(discussed in detail in Section 4.1 Previous work);[89-93] however, no study to date has conclusively associated rare variants in IHH genes with self-limited delayed puberty.

Despite the many promising candidate genes and genetic loci identified through case-reports, phenotypic similarities between self-limited delayed puberty and known genetic disorders, and genome-wide association and linkage studies in individuals with self-limited puberty, the exact causal variants and genes underlying delayed puberty remain unknown.

1.7 Connections between self-limited delayed puberty and IHH

Several observations suggest that there are common pathophysiologic mechanisms that link self-limited delayed puberty and isolated IHH. Individuals with delayed puberty and isolated IHH can co-exist in the same pedigree,[38, 44, 62, 64, 65, 94] and prior studies have reported that over 10% of IHH patients have relatives with a history of delayed puberty, compared to 2.5% of the general population.[95, 96] In addition, one distinction between the phenomenon of "reversal" of IHH and self-limited delayed puberty is the timing of pubertal initiation: after the age of 18 years in IHH with reversal and before age 18 years in self-limited delayed puberty. The specific phenotypic resemblance between self-limited delayed puberty and IHH with reversal suggests that variants in *TAC3* and *TACR3*, which has been specifically associated with IHH with reversal, may also contribute to the pathogenesis of self-limited delayed puberty, but prior investigations have not conclusively demonstrated such a link.[92, 93] Despite these initial findings, our observations suggest that, at least in some cases, self-limited delayed puberty and isolated IHH may share an underlying pathophysiology.

In this thesis, we have tested the hypothesis that there is overlap between the genetics of self-limited delayed puberty and that of isolated IHH[†] using two approaches. First, we examined pedigrees with an IHH proband known to carry a potentially pathogenic variant in an IHH gene to determine whether family members with self-limited delayed puberty were more likely than those with normal pubertal timing to share the proband's variant. We also examined probands with delayed puberty (and no family history of IHH) to test whether they were more likely than controls to harbor potentially pathogenic variants in IHH genes, with a specific focus on *TAC3* and *TACR3* given the association of these genes with IHH with reversal.

2 MATERIALS AND METHODS:

All studies were approved by the Institutional Review Boards (IRBs) of Massachusetts General Hospital (MGH), Boston Children's Hospital (BCH), and Rainbow Babies and Children's Hospital (RB&C), and written informed consent was obtained from all study participants.

2.1 Pedigrees with an IHH proband

2.1.1 STUDY PARTICIPANTS

IHH individuals were either patients at MGH or referred by their physicians to MGH to participate in genetic studies. IHH was defined as low sex-steroid levels (testosterone ≤ 100 ng/dL in men; estradiol ≤ 20 pg/mL in women) in the setting of inappropriately normal or low

[†]Given the severe phenotypes associated with variants in syndromic IHH genes and the unlikely possibility that individuals with self-limited delayed puberty would harbor variants in these genes without additional phenotypes, this thesis focuses on only the isolated IHH genes (Figure 2).

gonadotropin levels at age ≥18 years in the absence of other identifiable causes of hypogonadotropic hypogonadism.[27]

Family members in IHH pedigrees were evaluated by a staff member of the MGH Reproductive Endocrine Unit through standardized questionnaires, interviews, and/or review of medical records. Self-limited delayed puberty was defined in this cohort as 1) spontaneous menarche between the ages of 15 and 18 years for girls, initiation of testicular growth between the ages of 14 and 18 years for boys, reported or documented diagnosis of delayed puberty by a physician, and/or self-reported continual height growth past age 16 years, 2) no report of impaired reproductive endocrine function in adulthood, and 3) absence of identifiable underlying causes of delayed puberty. Family members who reported normal pubertal development were used as controls.

2.1.2 GENETIC STUDIES

Genomic DNA was extracted from peripheral blood samples or saliva of all study participants. IHH probands had previously been sequenced for variants in 13 IHH genes: *FGF8*, *FGFR1*, *GNRH1*, *GNRHR*, *HS6ST1, KAL1*, *KISS1*, *KISS1R*, *NELF, PROK2*, *PROKR2*, *TAC3*, and *TACR3*. Sequence variants were characterized as potentially pathogenic variants by the following criteria: (i) variants with minor allele frequency < 0.1% the 1000 Genomes Project[97] and National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project (NHLBI GO ESP);[98] (ii) nonsense, frameshift, or splice-site altering variants, and nonsynonymous missense variants predicted to be deleterious in ≥ 3/5 *in silico* prediction programs (PANTHER,[99] PolyPhen2,[100] SIFT,[101] pMUT,[102] and MutationTaster[103]). Whenever available, previous functional studies of these variants demonstrating loss of function *in vitro* provided additional evidence of pathogenicity. For each IHH proband with an identified potentially pathogenic variant, family members for whom DNA was available were screened for

the same variant in *FGFR1*, *HS6ST1, KAL1*, *PROKR2*, *TAC3*, and *TACR3* by PCR amplification and Sanger sequencing as previously described.[38, 40, 41, 62, 94]

2.2 Delayed Puberty Probands

2.2.1 STUDY PARTICIPANTS

Delayed puberty participants in the study were either patients at MGH, BCH, or RB&C, or referred to MGH to participate in genetic studies. Some of the delayed puberty subjects from BCH and RB&C were studied in previous reports on the diagnoses and characteristics of adolescents with delayed puberty,[26] familial aggregation and inheritance patterns in self-limited delayed puberty pedigrees,[69] and the role of sequence variation in IHH genes in pubertal timing.[89, 91] Self-limited delayed puberty was defined in this cohort as 1) absence of spontaneous thelarche by age 13 years and spontaneous menarche by age 15 years for girls and testicular length <2.5 cm or volume <4 mL at age 14 years or older for boys, 2) spontaneous pubertal development prior to age 18 years as evidenced by thelarche and/or spontaneous menarche in girls and testicular length ≥ 2.5 cm or testicular volume ≥ 4 mL in volume in boys, and 3) evidence of a normal rate of pubertal progression and/or achievement of normal or near-normal adult sex-steroid levels and/or testicular volume in boys, and 4) absence of identifiable underlying causes of delayed puberty. Controls were 33,855 non-Finnish European individuals from the Exome Aggregation Consortium (ExAC).[104]

2.2.2 GENETIC STUDIES

Genomic DNA was extracted from peripheral blood samples or saliva of all study participants. Control data were accessioned through the Exome Aggregation Consortium browser. Whole-exome sequencing, variant calling, and annotation were performed as described below. Variants were filtered using the following parameters: 1) present in one of the following

IHH genes: *DUSP6*, *FEZF1*, *FGF8*, *FGF17*, *FGFR1*, *FLRT3, GNRH1, GNRHR*, *HS6ST1*, *IL17RD*, *KAL1*, *KISS1*, *KISS1R*, *NELF, PROK2, PROKR2, SEMA3A*, *SPRY4*, *TAC3*, *TACR3*, and *WDR11*; 2) quality designation of PASS; 3) variant frequency within the delayed puberty or control cohort of < 20% (to exclude sequencing artifacts); and 4) a potentially pathogenic variant as defined above.

Whole-exome sequencing:

Library construction was performed as previously described with the following modifications: initial genomic DNA input into shearing was reduced from 3µg to 10-100ng in 50µL of solution.[105] For adapter ligation, Illumina paired end adapters were replaced with palindromic forked adapters, purchased from Integrated DNA Technologies, with unique 8 base molecular barcode sequences included in the adapter sequence to facilitate downstream pooling. With the exception of the palindromic forked adapters, the reagents used for end repair, A-base addition, adapter ligation, and library enrichment PCR were purchased from KAPA Biosciences in 96-reaction kits. In addition, during the post-enrichment SPRI cleanup, elution volume was reduced to 20µL to maximize library concentration, and a vortexing step was added to maximize the amount of template eluted.

After library construction, hybridization and capture were performed using the relevant components of Illumina's Rapid Capture Exome Kit and following the manufacturer's suggested protocol, with the following exceptions: first, all libraries within a library construction plate were pooled prior to hybridization. Second, the Midi plate from Illumina's Rapid Capture Exome Kit was replaced with a skirted PCR plate to facilitate automation. All hybridization and capture steps were automated on the Agilent Bravo liquid handling system.

After post-capture enrichment, library pools were quantified using quantitative PCR (automated assay on the Agilent Bravo), using a kit purchased from KAPA Biosystems with

probes specific to the ends of the adapters. Based on qPCR quantification, libraries were normalized to 2 nM, then denatured using 0.1 N NaOH on the Perkin-Elmer MiniJanus. After denaturation, libraries were diluted to 20 pM using hybridization buffer purchased from Illumina.

Cluster amplification of denatured templates was performed according to the manufacturer's protocol (Illumina) using HiSeq v3 cluster chemistry and HiSeq 2000 or 2500 flowcells. Flowcells were sequenced on HiSeq 2000 or 2500 using v3 Sequencing-by-Synthesis chemistry, then analyzed using RTA v.1.12.4.2 or later. Each pool of whole exome libraries was run on paired 76 bp runs, and an 8 base index sequencing read was performed to read molecular indices, across the number of lanes needed to meet coverage for all libraries in the pool.

Exome sequence data processing was performed using the Genome Analysis Toolkit (GATK).[106] Single-nucleotide polymorphism and insertion/deletion discovery and genotyping was performed using standard filtering parameters or variant quality score recalibration according to GATK Best Practices recommendations.[107, 108]

Annotation of variants was performed with SnpEff version 2.0.5.[109]

2.3 Statistical analysis:

For analysis of pedigrees with an IHH proband, the expected percentage of family members sharing an IHH proband's variant under the null hypothesis that the variant has no effect on pubertal phenotype was calculated according to the following formula: [(75% * number of 1st degree relatives in pedigrees in which the IHH proband had 2 variants) + (50% * number of 1st degree relatives in pedigrees in which the IHH proband had 1 variant) + (25% * number of 2nd degree relatives) + (12.5% * number of 3rd degree relatives)] / [total number of family members]. The probability of observing the observed results was determined by performing 10^6 random computer simulations under the null hypothesis and tallying the number of simulations that

produced results with a deviation from expected that was equal to or greater than that of the observed results (examining the proportion of family members with delayed puberty who shared the proband's variant, the proportion of unaffected family members, or the difference between these two proportions).

For analysis of delayed puberty probands, frequencies of potentially pathogenic variants between cases and controls were compared using Fisher's exact test. Comparisons of ages at pubertal milestones were performed using Student's t-test. All tests were two-sided, and a *P*-value < 0.05 was considered statistically significant.

3 RESULTS:

We analyzed two cohorts of subjects with self-limited delayed puberty: family members of an IHH proband who themselves had delayed puberty, and individuals with delayed puberty and no family history of IHH.

3.1 Pedigrees with an IHH Proband

3.1.1 ASCERTAINMENT OF PEDIGREES

Figure 3 summarizes the process of identifying pedigrees with an IHH proband with a potentially pathogenic variant in an IHH gene and family members with delayed puberty. Of 1,354 IHH probands in the Massachusetts General Hospital Reproductive Endocrine Unit (MGH REU) research database, 148 had pedigrees with one or more family members noted to have delayed puberty. In 131 of these pedigrees, the proband with IHH had previously been screened for exonic sequence variants in one or more of thirteen IHH genes, and 59 were found to have such a sequence variant with an initial rarity cutoff of a < 1% minor allele frequency in both the 1000 Genomes Project and the NHLBI GO ESP (See *Materials and Methods*). DNA was

available for family members with delayed puberty in 39 of these pedigrees, and review of records revealed that in 23 of these pedigrees the family members recorded as having delayed puberty met the criteria for self-limited delayed puberty used for this study (Figure 3). Sequence variants identified in the IHH probands were classified as "potentially pathogenic variants" based on their rarity (with a more stringent cutoff of a 0.1% minor allele frequency) and predicted pathogenicity in 18 of those 23 pedigrees (see *Materials and Methods*).

In pedigrees in which the IHH proband had a candidate variant in *KAL1*, an X-linked gene, only female family members were included in the analysis because pathogenic variants in *KAL1* are highly penetrant for IHH in men;[62] this led to removal of one pedigree in which DNA was available only for male family members, as any males with a pathogenic variant was highly likely to have an IHH phenotype. Two additional pedigrees in which the IHH probands had biallelic variants in *GNRHR* and DNA was available for only the proband's parents were removed, as both parents were assumed to carry one such variant. Of the 15 pedigrees that met criteria for inclusion in this analysis, one had an IHH proband with two potentially pathogenic variants (in *TAC3* and *HS6ST1*) (Figure 4); all other probands had a single potentially pathogenic variant in an IHH gene (Table 1).

Within these 15 pedigrees, DNA was available from 19 family members with self-limited delayed puberty and 33 family members with normal pubertal timing. No single pedigree contributed disproportionately: 11 pedigrees contributed one family member with delayed puberty each; and the remaining 4 each contributed two family members with delayed puberty. All pedigrees contributed 0-3 unaffected family members, except for one pedigree that contributed 6 (Table 1). To rule out differences in genetic relatedness as a potential confounding factor, the degree of relation of delayed puberty and unaffected family members to the IHH proband was assessed across the pedigrees. Of the delayed puberty and unaffected family

members, 74% and 79% were first-degree relatives, 21% and 18% were second-degree relatives, and 5% and 3% were third-degree relatives, respectively (Figure 3). Based on these degrees of relatedness, under the null hypothesis that the proband's variant has no effect on pubertal phenotypes in family members, ~44% of delayed puberty family members and ~44% of unaffected family members would be expected to share the IHH proband's variant.

3.1.2 GENETIC VARIANTS

Targeted sequencing was performed to determine whether delayed puberty family members and unaffected family members harbored the same variant as the IHH proband. The potentially pathogenic variants found in the IHH probands were shared by 53% (10/19) of delayed puberty family members compared to 12% (4/33) of unaffected family members (P = 0.003, determined by 10^6 random computer simulations under the null hypothesis as described above). This difference was primarily due to fewer unaffected family members sharing the IHH proband's variant than expected if the variant had no effect on pubertal timing (12% observed vs. 44% expected, $P < 0.0001$). There was also a suggestion of more delayed puberty family members sharing the IHH proband's variant than expected (53% observed vs. 44% expected), though this difference was not statistically significant ($P = 0.3$).

The genetic variants found to be shared between IHH probands and family members with delayed puberty were found in *FGFR1* (7 subjects from 6 pedigrees), *KAL1* (2 female subjects from 1 pedigree), and *TAC3* (1 subject). Of the 10 variants identified, 6 led to premature termination (*FGFR1* p.Q680X, p.R622X x2, and p.S346fsX18; *KAL1* p.R457X x2), 1 was a missense change previously shown to be loss-of-function *in vitro* (*FGFR1* p.Y99C),[65] and 3 were missense variants predicted to be pathogenic by 3 or more of 5 *in silico* prediction programs (*FGFR1* p.E274G and p.I639T; *TAC3* p.R80S) (Table 1).

In 2 of these pedigrees, the IHH proband's variant was also found in family members with normal pubertal timing (*FGFR1* p.Y99C; *KAL1* p.R457X) (Figure 4). In addition, in 2 pedigrees, the IHH proband's variant was shared only with unaffected family members. One of these two variants led to premature termination (*TACR3* p.W208X), and the other was previously shown to be loss-of-function *in vitro* (*PROKR2* p.S188L) (Table 1).[94]

3.1.3 PHENOTYPES

Of the 19 delayed puberty family members, 8 were women and 11 were men (Table 2). For the women, the average age of menarche (by self-report) was 15.9 years with a range of 15-17.5 years. For the men, the age of initiation of testicular growth (also by self-report) was available for 6 subjects, with an average of 15.3 years and a range of 14-16 years. There were no apparent differences in clinical characteristics (e.g., age of menarche, age of testicular growth) between the 10 delayed puberty family members with a potentially pathogenic variant in an IHH gene and the 9 without such a variant (Table 2).

3.2 Delayed Puberty Probands

Because IHH is a rare disease, the association of potentially pathogenic variants in IHH genes with delayed puberty in relatives of IHH probands could be unique to these special cases. To determine whether variants in IHH genes contribute to self-limited delayed puberty outside the context of pedigrees with an IHH proband, we used whole-exome sequencing to screen for sequence variants in 21 IHH genes in 56 subjects with delayed puberty, no family history of IHH, and self-reported European ancestry; 21 were referred to the MGH REU for clinical care and/or research studies, and 35 were patients at BCH and RB&C. We compared the frequency of potentially pathogenic variants in delayed puberty subjects to that of 33,855 non-Finnish European controls from ExAC.[104] Because individual level data was not available in ExAC, each

allele with a potentially pathogenic variant was counted as a single individual with such a variant.

3.2.1 GENETIC VARIANTS

Potentially pathogenic variants in IHH genes were identified in 14.3% (8/56) of delayed puberty probands compared to 5.6% (1,907/33,855) of controls ($P = 0.01$) (Table 3). Heterozygous variants identified in delayed puberty probands were found in *GNRHR* (n = 1, 1.8% of all delayed puberty subjects), *IL17RD* (n = 3, 5.4%), *SEMA3A* (n = 1, 1.8%), *TAC3* (n = 2, 3.6%), and *TACR3* (n = 1, 1.8%) (Tables 3 and 4). One potentially pathogenic variant identified in delayed puberty subjects led to premature termination (*IL17RD* p.W200X), one led to a splice-site change (*TAC3* g.18595G>T, in the donor splice site of exon 9), one was a missense variant previously shown to cause loss of function *in vitro* (*IL17RD* p.K131T),[44] and five were missense variants predicted to be deleterious in 3 or more of 5 *in silico* prediction programs (*GNRHR* p.L117R*; IL17RD* p.P191L; *TAC3* p.H83R; *TACR3* p.A171P; *SEMA3A* p.T717I) (Table 4). The *GNRHR* p.L117R, *IL17RD* p.K131T, and *SEMA3A* p.T717I variants identified in delayed puberty probands had been previously identified in subjects with IHH at the MGH REU and/or reported in prior studies (Table 4),[44, 110] as had 49 variants identified in 552 ExAC controls (Supplemental Table 1). The frequency of these known IHH variants was higher in the delayed puberty cohort compared to controls (5.4% vs. 1.6%, respectively), though this did not reach statistical significance ($P = 0.06$). All potentially pathogenic variants identified in ExAC controls are listed in Supplemental Table 1.

Given the association between IHH with reversal and *TAC3* and *TACR3*,[34] we specifically tested whether potentially pathogenic variants in these genes were enriched in subjects with self-limited delayed puberty compared to controls. Indeed, 5.4% (3/56) of delayed

puberty probands were found to harbor such variants in *TAC3* or *TACR3* compared to 0.3% (87/33,855) of controls ($P = 0.0005$) (Table 3).

The gene with the largest number of potentially pathogenic variants in delayed puberty probands was *IL17RD*, a modulator of signaling by the fibroblast growth factor receptor, with variants found in 3 delayed puberty subjects (5.4%) compared to 0.7% (231/33,855) of control subjects (Table 3).

3.2.2 PHENOTYPES

For women in the delayed puberty cohort for whom the age of menarche was documented (12 of 15 women), the average age of menarche was 16.0 years with a range of 15-18 years. For men for whom the age at which testicular size reached the pubertal range (≥ 4 mL in volume or ≥ 2.5 cm in length) was documented (30 of 41 men), the average age of reaching a pubertal testicular size was 15.5 years with a range of 14-17 years (Table 5). There were no apparent differences in clinical characteristics (e.g., age of menarche, age of testicular growth) between subjects recruited from BCH/RB&C and those from the MGH REU (Table 6). There were also no apparent differences in clinical characteristics between subjects with potentially pathogenic variants in IHH genes and subjects without such variants (Table 5). Of the 8 subjects with delayed puberty who had potentially pathogenic variants in IHH genes, 4 subjects were from the BCH/ RB&C cohort (11% of the cohort of 35), and 4 subjects were from the MGH REU cohort (19% of the cohort of 21). The slightly higher frequency of potentially pathogenic variants observed in the MGH REU cohort was not statistically significant ($P = 0.5$).

4 DISCUSSION:

We have found that subjects with self-limited delayed puberty are more likely than control subjects to carry potentially pathogenic variants in IHH genes, which are critical to the

development and function of the GnRH neuronal network. By implicating these genes in the pathogenesis of delayed puberty, our findings confirm the genetic basis of delayed puberty that had been suggested by previous studies that demonstrated its highly familial nature.[69, 70] Furthermore, the genetic link between delayed puberty and IHH suggests that the common, self-limited condition of delayed puberty and the rare, often permanent disorder of IHH share underlying pathophysiologic mechanisms.

4.1 Previous work

Several prior studies, some conducted by members of the MGH REU, have tested the hypothesis that either common or rare variants in IHH genes contribute to the pathogenesis of delayed puberty. These studies have examined both rare and common variants in *GNRH1* and *GNRHR* as well as common haplotypes around these genes,[89, 90] common variants in a panel of 10 IHH genes (*FGFR1*, *GNRH*, *GNRHR*, *GPR54*, *KAL1*, *KISS1*, *LEP*, *LEPR*, *PROK2*, and *PROKR2)*,[91] variants specifically associated with IHH in *KAL1*, *GNRHR*, and *FGFR1*,[91] and rare variants in *FGFR1*, *GNRHR*, *TAC3*, and *TACR3*.[92, 93] None of these studies demonstrated a statistically significant association between any of these variants and delayed puberty, though in one study a heterozygous nonsense variant in *GNRHR* (p.F309X) segregated with the delayed puberty phenotype in a pedigree,[92] and in another, a missense variant in *TACR3* (p.A449S), predicted to be benign on *in silico* analysis, was identified in a delayed puberty subject but not in controls.[93] Thus, though these studies have identified occasional rare variants in IHH genes in delayed puberty individuals, no study to date has conclusively associated rare variants in IHH genes with self-limited delayed puberty. Given the similarities between IHH and self-limited delayed puberty, the failure to demonstrate such an association was puzzling. The expansion of the IHH gene panel over the years and the use of whole-exome sequencing in this study to screen

simultaneously for variants in all genes associated with isolated IHH have now allowed us to demonstrate an enrichment of rare variants in IHH genes in subjects with self-limited delayed puberty.

4.2 Genetic and phenotypic variability in self-limited delayed puberty and IHH

Our results also highlight the variability in both the genetic variants and phenotypes associated with variants in IHH genes. All potentially pathogenic variants identified in delayed puberty subjects were heterozygous. While IHH associated with variants in *FGFR1* and *FGF8* have been shown to predominantly follow an autosomal-dominant mode of inheritance,[54, 55] heterozygous variants in the remaining genes associated with IHH would not be necessarily be expected to lead to a disease phenotype. However, heterozygous variants in *TACR3*, *GNRHR, GNRH1*, and *PROKR2*,[40, 58, 64, 94] as well as the secreted ligand *PROK*2[111] have been clearly associated with the reproductive endocrine dysfunction of IHH.[111, 112] One possibility is that these variants act in a dominant manner, as some variants in *GNRHR* have been shown to regulate wild-type receptors in a dominant-negative manner.[113] Another possibility is that these individuals have another defect in a yet-to-be identified gene critical to the function of the GnRH network, as IHH has been suggested to have an oligogenic inheritance pattern in some instances.[67] A third possibility, which stems from the variability in phenotypes associated these variants seen in this study, is variable penetrance and expressivity. In a previous study on *FGFR1*, the same heterozygous variants were associated with IHH, delayed puberty, and normal reproductive endocrine function within the same pedigree.[32]

Similarly in our study, potentially pathogenic variants in multiple IHH genes were found not only in subjects with delayed puberty but also in controls. Because limited phenotypic data was available for the ExAC controls, it is possible that some controls harboring variants in IHH

genes in fact had delayed puberty. Furthermore, though these variants were predicted to be deleterious by multiple computer prediction programs, it remains possible that some of these variants have little to no impact on the function of the encoded proteins. As discussed above, these variants in IHH genes may also exhibit variable penetrance and expressivity. Indeed, in two of the IHH pedigrees analyzed in this study, the same heterozygous variant was found in the IHH proband, family members with delayed puberty, and family members with normal pubertal timing. However, in our analysis of pedigrees with an IHH proband, few unaffected family members shared the proband's variant, suggesting that these IHH gene variants tend to produce either delayed puberty or IHH. The genetic and phenotypic variability associated with variants in IHH genes complicates the interpretation of genetic testing for these conditions, and future studies are needed to more clearly define the genetic variants and their associated phenotypes to determine the clinical utility of genetic testing.

4.3 A critical role for the neurokinin B pathway in pubertal timing

Our results provide further evidence for the association between rare variants in the neurokinin B signaling pathway and delayed emergence of reproductive endocrine function, as rare variants in *TAC3* and *TACR3* have now been identified in individuals with self-limited delayed puberty as well as in individuals with IHH with reversal. Interestingly, a common genetic variant near *TACR3* has been shown to be associated with variation in the normal timing of puberty.[84, 85] Thus, disruption of the neurokinin B pathway can lead to late-normal pubertal timing, frankly delayed puberty, or even more severely delayed sexual maturation that does not occur until after the age of 18 years, firmly demonstrating a critical role for the neurokinin B pathway in influencing pubertal timing.

4.4 An emerging role for IL17RD in delayed puberty

We also identified several potential pathogenic variants in *IL17RD*, a modulator of the FGF8/FGFR1 signaling pathway, which has been suggested to have a role in the fate specification of GnRH neurons.[44] A recent study identified a heterozygous variant in *IL17RD* (p. S468L) in both an IHH proband and a parent with delayed puberty and another variant in *IL17RD* (p. P577Q) in the homozygous state in an IHH proband and in the heterozygous state in one parent who had delayed puberty.[44] These findings and our current observations collectively suggest that heterozygous variants in *IL17RD* may produce a partial functional defect resulting in reproductive endocrine phenotypes of varying severity.

4.5 Refining the physiological model of puberty

IHH genes have been broadly classified based on their roles in either the neurodevelopmental ontogeny or neuroendocrine physiology of GnRH neurons.[1] It is fairly plausible that *TAC3* and *TACR3*, neuroendocrine genes that appear to regulate GnRH neuronal function, could function as part of the hypothetical "switch" that activates the GnRH neuronal network to initiate puberty. Defects in either *TAC3* or its receptor *TACR3* could disrupt the autoregulatory positive feedback loop in KNDy neurons that modulates kisspeptin signaling. Abnormal or dampened kisspeptin secretion could in turn disrupt the pulsatile GnRH secretion critical to the activation of HPG axis at puberty. It is less clear, perhaps, how delayed puberty could be caused by deleterious variants in *IL17RD*, a presumed neurodevelopmental gene involved in the initial fate specification of GnRH neurons.[44] One potential mechanism is that *IL17RD* may function not only in the development of GnRH neurons but also in other yet-to-be identified pathways critical for activating GnRH neurons at puberty. Another possibility is that *IL17RD* has some positive feedback role, whether direct or indirect, in the known pathways that

initiate puberty similar to neurokinin B, which remain poorly understood. A third possibility stems from the fact that the hormonal changes underlying puberty can be detected well before the appearance of physical signs of puberty: in boys, nighttime LH secretion has been shown to occur 1-2 years before testicular enlargement can be appreciated.[3] Thus, even if the first activation of GnRH neurons occurs at a normal time, impaired activity of the GnRH neuronal network due to deleterious variants in a neurodevelopmental gene could result in a longer delay between the activation of this diminished GnRH neuronal network and the appearance of the physical changes that are used clinically to mark the start of puberty.

4.6 Growth delay in self-limited delayed puberty

Self-limited delayed puberty has traditionally been described as occurring in the context of CDGP, with associated delays in childhood growth and skeletal maturation. However, in one study, over 50% of individuals with delayed puberty did not exhibit slow prepubertal growth.[114] This observation suggests two distinct pathophysiological mechanisms for delayed puberty, one associated with delays in prepubertal growth and the second with isolated pubertal delay. As the GnRH neuronal network is relatively quiescent during childhood, defects in this network would not be expected to cause prepubertal growth delay, but rather pubertal delay in the context of a normal childhood growth pattern. Longitudinal growth data were not available for most of our delayed puberty subjects, and future work will determine whether delayed puberty associated with IHH gene variants indeed occurs in the absence of delayed prepubertal growth.

4.7 Strengths and limitations

Strengths of our study include the use of exome sequencing to analyze the largest IHH gene panel (21 genes) in a delayed puberty cohort to date, as well as the use of a large, ethnically

matched control cohort. Limitations of our study include the relatively small size of the cohorts, which were mostly underpowered to implicate specific genes in the pathogenesis of self-limited delayed puberty, though we have identified candidate genes (*TACR3*, *TAC3*, and *IL17RD*) for future genetic studies of delayed puberty. Another limitation is the difference in whole-exome capture techniques used for the delayed puberty cohort and the ExAC controls, which could potentially result in differences in variant detection. However, all potentially pathogenic variants detected in the delayed puberty cases were in genomic regions with adequate coverage in ExAC. In addition, because individual level genotype data was not available for the ExAC cohort, each allele with a potentially pathogenic variant was counted as a single individual with such a variant. As it is possible that a single individual may have had more than one potentially pathogenic variant in an IHH gene, we are likely to have overestimated the actual number of individuals with potentially pathogenic variants. However, this would have reduced our power to detect differences and, thus, does not invalidate our positive findings. A fourth limitation is selection bias. Because the MGH REU is a referral center for IHH, the referred delayed puberty cases may have been more severe and, thus, not representative of delayed puberty more generally. However, no significant differences were found in clinical characteristics or the frequency of potentially pathogenic variants between the MGH and BCH/RB&C cohorts. Future studies are needed to determine the degree to which variants in IHH genes contribute to delayed puberty in more general populations.

4.8 Linking the genetics of rare and common disorders

Our findings provide evidence for a genetic link between IHH, a rare disease, and self-limited delayed puberty, a common disorder. Mendelian diseases or syndromes have been traditionally attributed to rare variants with large effects on phenotype. In contrast, more

common variations in phenotype in the general population with high heritability, such as height, are thought to be due to common variants with small but additive effects.[115] However, the genes underlying the severe, rare diseases have provided insight into the genetics of more common heritable phenotypes. Genetic links between rare and common disease entities or clinical traits have been previously demonstrated between syndromes with short stature or somatic overgrowth and height variation in the general population (*HMGA2*)[116-118] (*GDF5*),[115, 119] neonatal diabetes and type 2 diabetes (*KCNJ11)*,[120-122] and familial dysbetalipoproteinemia and elevated fasting blood lipids (*APOE*).[123, 124] In reproductive endocrinology, a genetic link has been suggested between IHH and hypothalamic amenorrhea, a type of reproductive endocrine dysfunction that occurs in the setting of excessive exercise, nutritional deprivation, psychological distress, or other stressor.[125] Our study serves as an additional example of how rare variants with moderate to large effects in genes that underlie a rare genetic disease can also contribute to the genetics of a more common counterpart.

5 SUMMARY:

Puberty remains an intriguing scientific mystery, and the factors that initiate puberty and modulate its progression are still largely unknown. Self-limited delayed puberty has been shown to be highly heritable, and many candidate genes and genetic loci for the condition have been previously proposed. Despite numerous investigations of these genetic candidates, no specific variant or gene has been definitively associated with delayed puberty to date.

In contrast, multiple genes have been implicated in the rare, severe condition of idiopathic hypogonadotropic hypogonadism (IHH). Several observations have suggested a link between self-limited delayed puberty and IHH. Individuals with self-limited delayed puberty frequently exist in IHH pedigrees, and individuals with a subtype of IHH (IHH with reversal)

undergo spontaneous puberty in adulthood. Given these similarities between IHH and self-limited delayed puberty, we hypothesized that variants in IHH genes also contribute to the pathogenesis of self-limited delayed puberty.

To test our hypothesis, we analyzed the DNA sequences of two cohorts of subjects with self-limited delayed puberty: 1) family members of an IHH proband who themselves had delayed puberty, and 2) individuals with delayed puberty and no family history of IHH. In both analyses, we found that individuals with delayed puberty were significantly more likely to carry a potentially pathogenic variant in an IHH gene compared to controls.

For the first time, we have associated specific genes with self-limited delayed puberty. Our results highlight self-limited delayed puberty as an additional human disease model to decipher the intricacies of pubertal timing. Specifically, our study identifies *TAC3, TACR3*, and *IL17RD* as candidate delayed puberty genes. Future genetic studies may firmly establish these and other genes in the pathogenesis of delayed puberty, and functional studies will determine how these genes regulate pubertal timing.

More broadly, a better understanding of the genetics underlying diseases with abnormalities in pubertal timing may enable critical advances to determining the answer to the mystery of "What starts puberty?" Identifying and characterizing the genetic players in self-limited delayed puberty and IHH has the potential to create new opportunities to discover new and effective diagnostic and therapeutic tools for these and other reproductive endocrine disorders.

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8 TABLES AND FIGURES:

Table 1. Potentially Pathogenic Variants in IHH Probands and Family Members

	IHH Proband	Unaffected Family Members	Delayed Puberty Family Members	
Pedigree	Potentially Pathogenic Variant	N with a Potentially Pathogenic Variant/Total	N with a Potentially Pathogenic Variant/Total	Ref.
1	*FGFR1* p.Y99C	1/3	1/1	65†,54
2	*FGFR1* p.P366L	0/3	0/1	126
3	*FGFR1* p.Q680X	0/1	1/1	63, 65
4	*FGFR1* p.G237S	0/3	0/2	63†
5	*FGFR1* p.E274G	0/3	1/1	32
6	*FGFR1* p.W737R	0/2	0/1	—
7	*FGFR1* p.R622X	0/1	2/2	32, 54, 127
8	*FGFR1* p.G687R	0/6	0/1	—
9	*FGFR1* p.I639T	0/2	1/2	—
10	*FGFR1* p.S346YfsX18	0/1	1/1	—
11*	*HS6ST* p.R382W *TAC3* p.R80S	0/0	1/1	—
12	*KAL1* p.R457X	1/2	2/2	62
13	*PROKR2* p.S188L	1/2	0/1	94†
14	*PROKR2* p.R85C	0/1	0/1	94
15	*TACR3* p.W208X	1/3	0/1	40
TOTAL		4/33 (12%)‡	10/19 (53%)‡	

Abbreviation: —, none identified

* Only the *TAC3* p.R80S variant was shared by family members.

† Loss-of-function as indicated by significantly decreased performance in *in vitro* functional assays compared to wild-type

‡ $P = 0.003$ in the comparison of the number of unaffected vs. delayed puberty family members found to share the IHH proband's variant.

Table 2. Characteristics of Delayed Puberty Subjects in IHH Pedigrees

	All Delayed Puberty Subjects (N=19)				Delayed Puberty Subjects with Potentially Pathogenic Variants in IHH Genes (N=10)			
	Men (N=11)		Women (N=8)		Men (N=6)		Women (N=4)	
	Number or Mean (range)	N	Number or Mean (range)	N	Number or Mean (range)	N	Number or Mean (range)	N
Age at pubertal testicular size* (y)	15.3 (14-16)	6	—		15.4 (14-16)	3	—	
Age at growth spurt (y)	16.2 (14-18)	6			17.5 (17-18)	2		
Age at thelarche (y)	—		15.3 (15-16)	3	—		15	1
Age at menarche (y)	—		15.9 (15-17.5)	8	—		15.8 (15-16)	4
Spontaneous fertility (number of subjects)	6	†	6	†	4	†	2	†

Abbreviation: —, not applicable

* Testicular volume ≥ 4 ml or testicular length ≥ 2.5 cm

† Unknown number of subjects who sought fertility

Table 3. Potentially Pathogenic Variants by Gene in Delayed Puberty Probands

Gene	Number of Delayed Puberty Subjects (N=56) with a Potentially Pathogenic Variant	%	Number of Control Subjects (N=33,855) with a Potentially Pathogenic Variant	%	P*
DUSP6	0	0.0%	133	0.4%	1
FEZF1	0	0.0%	19	0.1%	1
FGF17	0	0.0%	13	0.0%	1
FGF8	0	0.0%	33	0.1%	1
FGFR1	0	0.0%	153	0.5%	1
FLRT3	0	0.0%	39	0.1%	1
GNRH1	0	0.0%	33	0.1%	1
GNRHR	1	1.8%	140	0.4%	0.2
HS6ST1	0	0.0%	69	0.2%	1
IL17RD	3	5.4%	231	0.7%	0.006
KAL1	0	0.0%	145	0.4%	1
KISS1	0	0.0%	6	0.0%	1
KISS1R	0	0.0%	51	0.2%	1
NELF	0	0.0%	89	0.3%	1
PROK2	0	0.0%	72	0.2%	1
PROKR2	0	0.0%	146	0.4%	1
SEMA3A	1	1.8%	257	0.8%	0.3
SPRY4	0	0.0%	62	0.2%	1
TAC3	2	3.6%	20	0.1%	0.0006
TACR3	1	1.8%	67	0.2%	0.1
WDR11	0	0.0%	129	0.4%	1
TOTAL	8	14.3%	1907	5.6%	0.01
TAC3+TACR3	3	5.4%	87	0.3%	0.0005

* *P*-values are not corrected for multiple comparisons.

Table 4. Potentially Pathogenic Variants in Delayed Puberty Probands

Potentially Pathogenic Variant	Evidence for Pathogenicity		Identified in IHH Subject(s) [Ref.]
	Severe Variant or LOF [Ref.]	*In silico* Predictions (Deleterious Predictions/Total Predictions)*	
GNRHR p.L117R		5/5	Yes[110]
IL17RD p.K131T	LOF[44]	3/4	Yes[44]
IL17RD p.P191L		4/4	No
IL17RD p.W200X	nonsense	—	No
SEMA3A p.T717I		3/5	Yes (Unpublished)
TAC3 p.H83R		5/5	No
TAC3 g.18595G>T	splice-site	—	No
TACR3 p.A171P		4/5	No

Abbreviation: LOF, loss-of-function as indicated by significantly decreased performance in in vitro functional assays compared to wild-type; Ref., reference; —, not tested

* Not all prediction programs gave predictions for all variants.

Table 5. Characteristics of Delayed Puberty Probands

	All Delayed Puberty Probands (N=56)				Delayed Puberty Probands with Potentially Pathogenic Variants in IHH Genes (N=8)			
	Men (N=41)		Women (N=15)		Men (N=7)		Women (N=1)	
	Mean (range)	N	Mean (range)	N	Mean (range)	N	Mean (range)	N
Age at pubertal testicular size* (y)	15.5 (14-17)	30	—		15.7 (15-16)	4	—	
Age at thelarche (y)	—		14.4 (14-15)	11	—		14	1
Age at menarche (y)	—		16 (15-18)	12	—		15	1

Abbreviation: —, not applicable; Ref., references

* Testicular volume ≥ 4 ml or testicular length ≥ 2.5 cm

Table 6. Sub-Cohort Characteristics of Delayed Puberty Probands

	BCH/ RB&C (N=35)				MGH REU (N=21)			
	Men (N=26)		Women (N=9)		Men (N=15)		Women (N=6)	
	Mean (range)	N	Mean (range)	N	Mean (range)	N	Mean (range)	N
Age at pubertal testicular size* (y)	15.3 (14-17)	22	—		15.9 (15-17)	8	—	
Age at thelarche (y)	—		14.3 (14-15)	9	—		14.5 (14-15)	2
Age at menarche (y)	—		15.9 (15-18)	7	—		16.2 (16-17)	5

Abbreviation: MGH REU, Massachusetts General Hospital Reproductive Endocrine Unit, BCH, Boston Children's Hospital; RB&C, Rainbow Babies and Children's Hospital, —, not applicable

* Testicular volume ≥ 4 ml or testicular length ≥ 2.5 cm

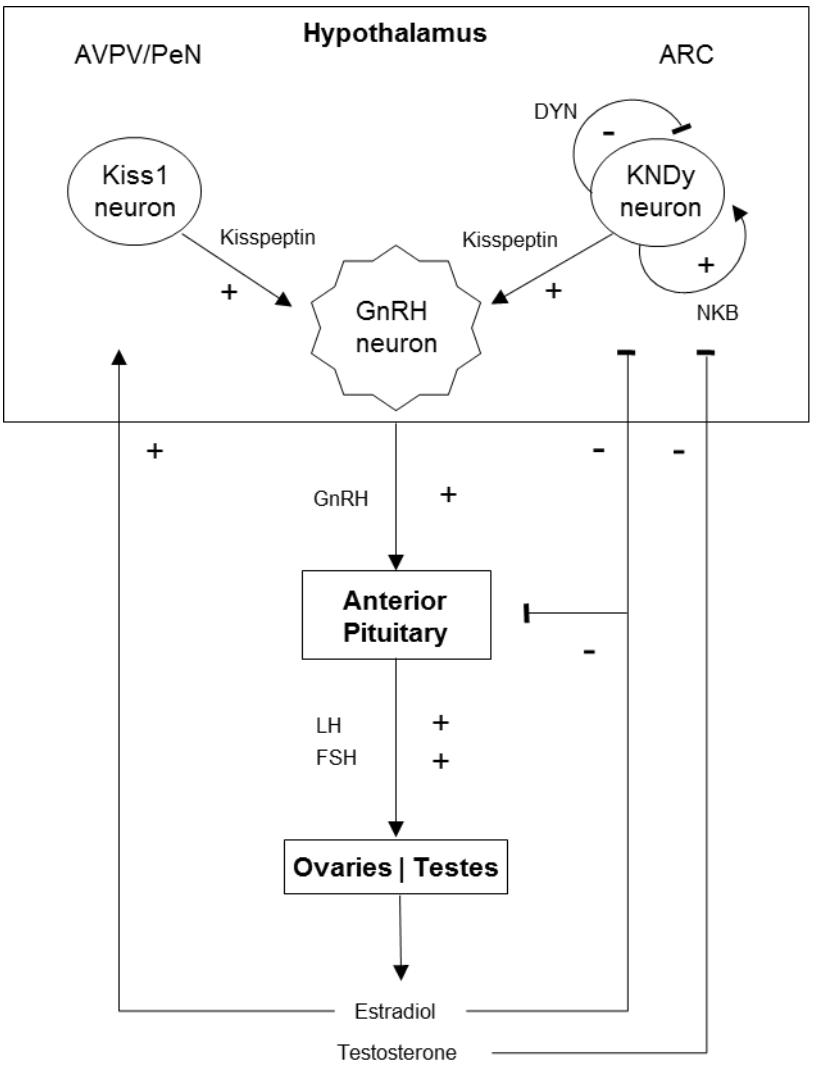

Figure 1. Function and regulation of the hypothalamic-pituitary-gonadal axis. Kiss1 neurons in the anteroventral periventricular nucleus/rostral periventricular nucleus (AVPV/PeN) and KNDy neurons in the arcuate nucleus (ARC) both secrete kisspeptin, which acts on GnRH neurons to secrete pulsatile gonadotropin-releasing hormone (GnRH). GnRH binds to receptors on the anterior pituitary to activate secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which act on the gonads to produce the sex steroids estradiol and testosterone. The sex steroids form negative feedback loops with the hypothalamus and pituitary to regulate GnRH and gonadotropin secretion. KNDy neurons secrete dynorphin A (DYN) and neurokinin B (NKB) in addition to kisspeptin, which are negative and positive autoregulators of kisspeptin signaling, respectively. The Kiss1 neurons in the AVPV/PeN are involved in positive feedback regulation by estradiol believed to mediate the gonadotropin surge that governs ovulation. Plus sign (+) indicates positive regulation, minus sign (-), negative regulation. Figure adapted from Navarro and Tena-Sempere et al. 2012.[7]

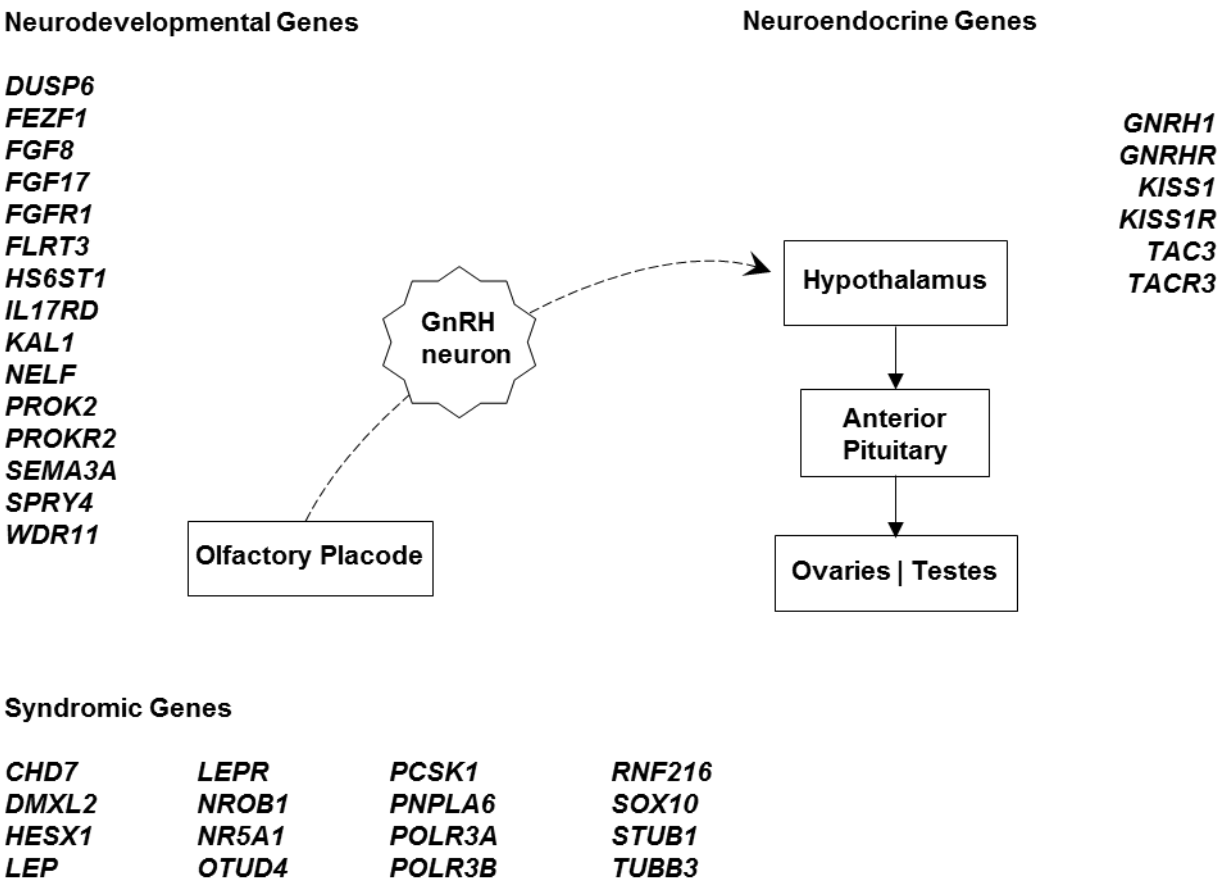

Figure 2. Genes implicated in idiopathic hypogonadotropic hypogonadism (IHH). In individuals with isolated IHH, variants have been identified in two main categories of genes: 1) Neurodevelopmental genes critical to the development and migration of GnRH neurons, and 2) Neuroendocrine genes critical to the regulation of GnRH secretion or action. In individuals with IHH in the context of a broader syndrome, variants have been identified in a wide variety of genes. The role that these syndromic genes play in the pathogenesis of IHH and the reproductive phenotypes associated with variants in these genes are still under investigation. Figure adapted from Bianca and Kaiser 2009.[56]

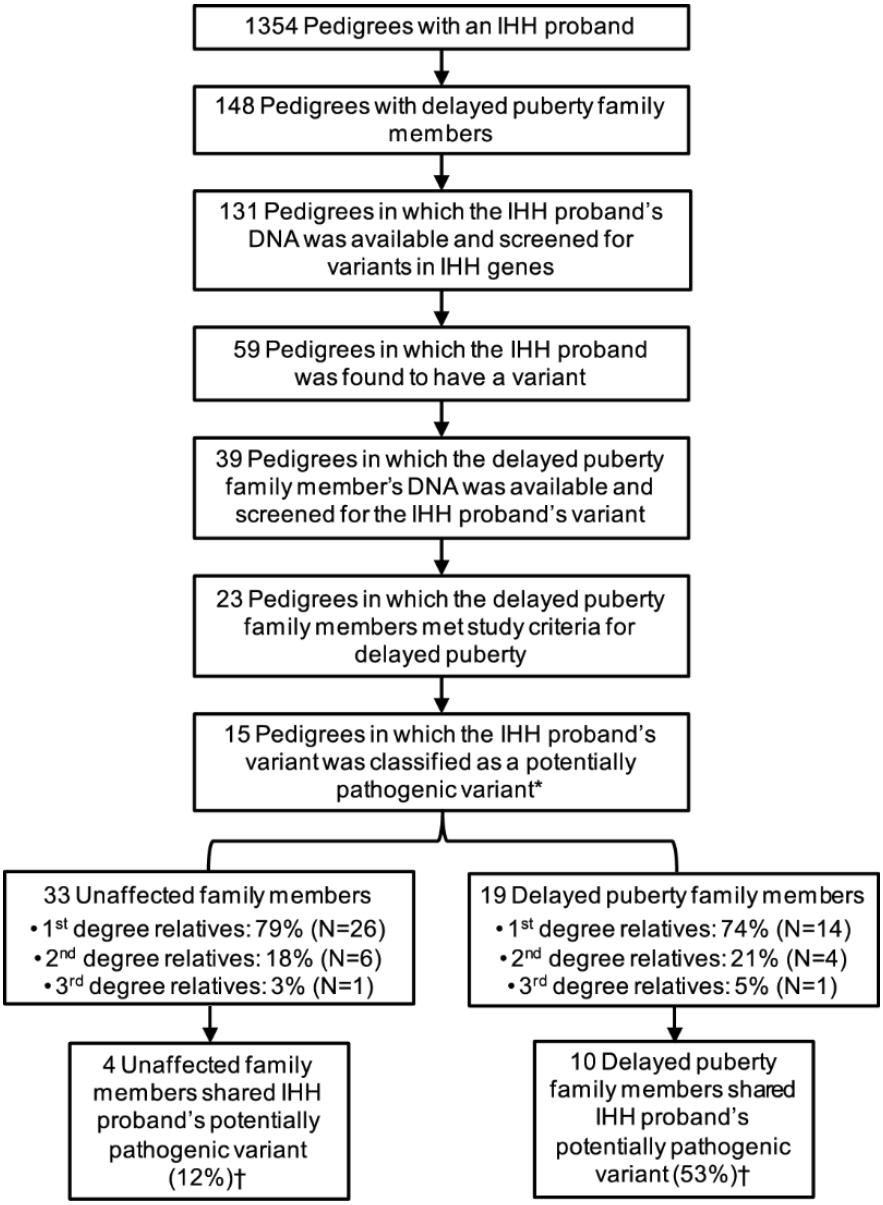

Figure 3. Ascertainment of family members with delayed puberty and normal pubertal timing in pedigrees with IHH probands. N, number of individuals specified in associated rectangle. *Three pedigrees were excluded: one in which the IHH proband had a potentially pathogenic variant in *KAL1* and DNA was available only for male family members, and two in which the IHH probands had biallelic variants in *GNRHR* and DNA was available for only the proband's parents. † $P = 0.003$ in the comparison of the number of unaffected vs. delayed puberty family members found to share the IHH proband's variant.

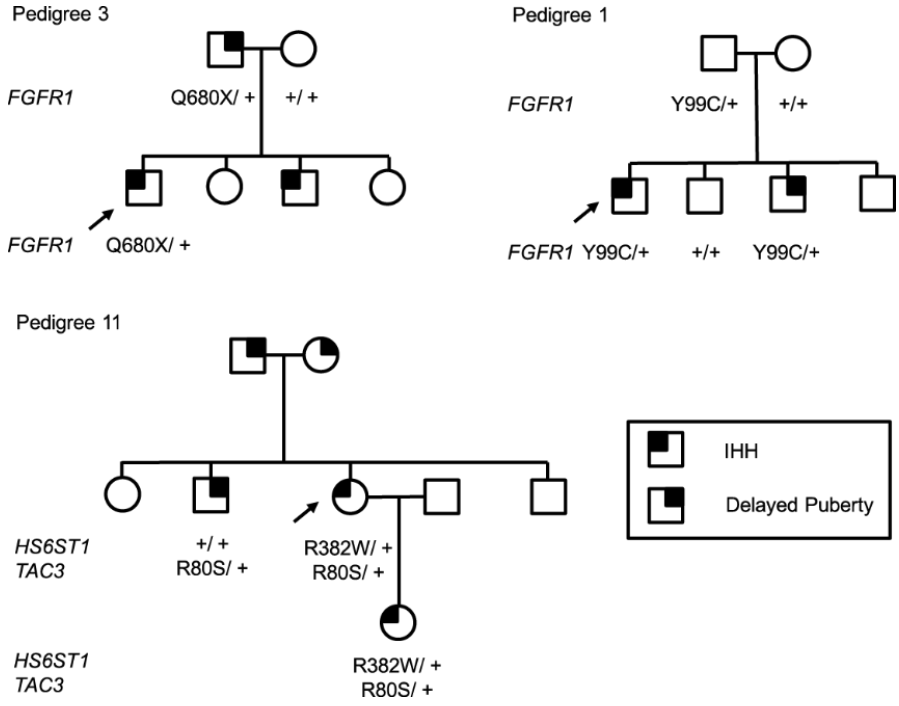

Figure 4. Examples of pedigrees with potentially pathogenic variants that segregate with IHH and delayed puberty (Pedigrees 3 and 11 from Table 1) and of a pedigree with a potentially pathogenic variant that does not (Pedigree 1). Arrows identify the IHH proband. Genotypes of subjects for whom DNA was available are listed below each subject's symbol. Plus sign (+) indicates the wild-type allele. Squares represent males, circles females.

Supplemental Table 1. Potentially Pathogenic Variants in ExAC Controls

Potentially Pathogenic Variant	Evidence for Pathogenicity		Identified in IHH Subject(s) [Ref.]	Number of Variant Alleles†
	Severe variant or LOF [Ref.]	*In silico* predictions (deleterious predictions/ total predictions)*		
DUSP6 p.T381A		4/5		1
DUSP6 p.L378P		4/4		1
DUSP6 p.N371H		3/4		3
DUSP6 p.C353R		4/5		2
DUSP6 c.1048delA: p.S350fs	frameshift	—		1
DUSP6 p.T346M		4/5	Yes[1]	25
DUSP6 p.S331F		5/5		1
DUSP6 p.S315L		4/5		1
DUSP6 p.H292R		4/5		1
DUSP6 p.E281A		3/5		2
DUSP6 p.A275S		3/5		1
DUSP6 p.P259T		5/5		1
DUSP6 p.L246P		4/5		1
DUSP6 p.Y235H		3/5		1
DUSP6 p.L214V		4/5		1
DUSP6 p.D187Y		3/4		1
DUSP6 p.S182F		4/4		80
DUSP6 p.R128W		4/4		8
DUSP6 p.S17R		3/4		1
FEZF1 p.Q397X	nonsense	—		1
FEZF1 c.1102_1103insT: p.H368fs	frameshift	—		1
FEZF1 p.Y359C		4/5		1
FEZF1 p.G350S		4/5		1
FEZF1 p.P343Q		4/5		1
FEZF1 p.P315T		4/5		1
FEZF1 p.H272Y		3/5		5
FEZF1 p.N270T		3/5		1
FEZF1 g.7057C>T	splice-site	—		1
FEZF1 p.C262X	nonsense	—		1
FEZF1 p.G251C		3/5		1
FEZF1 p.P79H		4/5		1
FEZF1 p.M74V		3/5		1
FEZF1 p.H66L		3/5		1
FEZF1 p.R35Q		4/5		1
FGF17 c.35_35del: p.L12fs	frameshift	—		1
FGF17 p.G25E		3/5		1
FGF17 p.N27Y		4/5		1

FGF17 p.G70R		4/5		2
FGF17 p.R72C		4/5		2
FGF17 p.D79Y		4/5		1
FGF17 p.G80C		4/5		1
FGF17 p.P120L		4/5		3
FGF17 p.R210G		5/5		1
FGF8 p.R222H		3/5		1
FGF8 p.R222C		5/5		6
FGF8 p.R219C		5/5		1
FGF8 p.T218M		3/5		2
FGF8 p.P215L		5/5		1
FGF8 p.F209L		3/5		1
FGF8 p.R208S		4/5		1
FGF8 p.E204K		4/5		1
FGF8 p.R198W		5/5		2
FGF8 p.P197L		4/5		1
FGF8 p.R188H		3/5		1
FGF8 p.R188C		5/5		2
FGF8 p.R176Q		5/5		1
FGF8 p.R176W		5/5		1
FGF8 p.R173H		5/5		1
FGF8 p.R173C		5/5		2
FGF8 p.N155S		3/5		1
FGF8 p.G123D		4/5		1
FGF8 p.R118Q		4/5		1
FGF8 p.V106L		4/5		1
FGF8 p.T78I		4/5		1
FGF8 p.Y72H		4/5		3
FGFR1 p.R822H		4/4		4
FGFR1 p.R822C		4/4		10
FGFR1 p.R821H		4/5		1
FGFR1 p.R821C		4/4		1
FGFR1 p.P811R		4/5		3
FGFR1 p.R809X	nonsense	—		1
FGFR1 p.C806X	nonsense	—		1
FGFR1 p.C806Y		5/5		1
FGFR1 p.P800L		3/4	Yes[2]	6
FGFR1 p.H798R		5/5		2
FGFR1 c.2370_2371del: p.S790fs	frameshift	—		1
FGFR1 p.T787M		5/5		6
FGFR1 p.R784Q		4/4		4
FGFR1 p.D782N		3/5		2
FGFR1 p.V758M		3/5		1
FGFR1 p.R756H		5/5		2
FGFR1 p.E728K		5/5		1
FGFR1 p.E715D		3/5		1
FGFR1 p.G703S		4/5	Yes[3]	3
FGFR1 p.R675P		5/5		3
FGFR1 p.D652N		5/5		1
FGFR1 p.N635I		4/4		1

FGFR1 p.R609Q		5/5		1
FGFR1 c.1818_1819insATCT TCAC: p.V607fs	frameshift	—		1
FGFR1 p.P591L		3/4		1
FGFR1 p.E571K		5/5		1
FGFR1 p.S565C		5/5		1
FGFR1 p.I560T		5/5		1
FGFR1 p.H541D		5/5		1
FGFR1 p.G539E		5/5		1
FGFR1 p.M532T		5/5		2
FGFR1 p.A520T		4/5	Yes[4]	1
FGFR1 p.M515T		5/5		1
FGFR1 p.R507H		3/5		8
FGFR1 p.P483R		4/5		1
FGFR1 p.P483S		4/5		1
FGFR1 p.R475Q		5/5		1
FGFR1 p.R470C		4/5		2
FGFR1 p.Y463C		4/5		1
FGFR1 p.V460F		4/5		1
FGFR1 p.P455A		3/5		1
FGFR1 p.R448Q		3/5		1
FGFR1 p.R448W		5/5	Yes[5]	1
FGFR1 p.R445Q		4/5		2
FGFR1 p.R445W		5/5		1
FGFR1 p.G440E		3/5		4
FGFR1 p.S439C		5/5		1
FGFR1 p.S436C		5/5		3
FGFR1 p.S434R		4/5		1
FGFR1 p.R425I		4/4		1
FGFR1 p.R424H		3/4		4
FGFR1 p.V358I		3/5		1
FGFR1 p.Q334X	nonsense	—		1
FGFR1 p.G301D		3/4		1
FGFR1 p.P252R		5/5		1
FGFR1 p.V248M		4/5		1
FGFR1 p.D246G		5/5		1
FGFR1 p.V232L		4/5		1
FGFR1 p.N227S		3/5		1
FGFR1 p.R189H		3/5		6
FGFR1 p.P158L		4/5		1
FGFR1 g.40490G>C	splice-site	—		1
FGFR1 p.T141R		3/4	Yes[6]	7
FGFR1 p.S135F		4/4		1
FGFR1 p.S134F		4/4		1
FGFR1 p.D130N		4/5		1
FGFR1 p.D129A		4/5	Yes[4]	3
FGFR1 p.T111I		3/5		1
FGFR1 p.R80H		3/5		2
FGFR1 p.R80C		3/5		1
FGFR1 p.R68W		4/5		1
FGFR1 p.R54C		3/5		2

FGFR1 g.28530G>T	splice-site	—		1
FGFR1 p.P28L		4/5		4
FGFR1 p.P23L		4/5		3
FGFR1 p.R22G		4/5		1
FGFR1 p.L18F		3/5		1
FGFR1 p.A16V		3/5		2
FGFR1 p.L13P		3/5		1
FGFR1 g.11299A>G	splice-site	—		1
FLRT3 p.Y636H		4/5		1
FLRT3 p.R634Q		4/4		4
FLRT3 p.L622P		3/4		1
FLRT3 p.T613I		4/4		1
FLRT3 p.N584K		4/5		1
FLRT3 p.Y565C		4/4		2
FLRT3 p.G534D		5/5		1
FLRT3 p.I533T		5/5		1
FLRT3 p.P481L		4/5		1
FLRT3 p.P471A		3/5		1
FLRT3 p.G379R		3/5		1
FLRT3 p.G331E		3/5		1
FLRT3 p.R317C		4/5		3
FLRT3 p.R303P		5/5		1
FLRT3 p.R303H		4/5		2
FLRT3 p.R275G		3/5		3
FLRT3 p.R234Q		3/5		1
FLRT3 p.R203C		4/5		1
FLRT3 p.L201V		3/5		1
FLRT3 p.S167G		4/5		1
FLRT3 p.L159F		4/5		1
FLRT3 p.I145T		4/5		1
FLRT3 p.L109F		4/5		1
FLRT3 p.L109V		4/5		3
FLRT3 p.L95X	nonsense	—		1
FLRT3 p.L90V		4/5		1
FLRT3 p.I70X	nonsense	—		1
FLRT3 p.I56M		3/5		1
GNRH1 p.K90N		4/5		18
GNRH1 p.Q89X	nonsense	—		1
GNRH1 p.R73X	nonsense	—	Yes[7]	1
GNRH1 p.C63Y		5/5		2
GNRH1 p.I48R		3/5		7
GNRH1 p.E39K		4/5		2
GNRH1 p.R36T		5/5		1
GNRH1 c.60dupC: p.C21fs	frameshift	—		1
GNRHR p.N305Y		4/5		1
GNRHR p.W291S		3/5		1
GNRHR p.T269M		4/5	Yes[8]	2
GNRHR p.R262W		5/5		1
GNRHR p.K217X	nonsense	—		1
GNRHR p.R240W		5/5		1
GNRHR p.I235N		4/5		1

GNRHR p.N231K		3/5		1
GNRHR p.N212K		4/5	Yes (Unpublished)	1
GNRHR p.C200Y	LOF[9-13]	5/5	Yes[9]	5
GNRHR p.G188V		3/5		1
GNRHR p.S187C		4/5		1
GNRHR p.A171T	LOF[13, 14]	4/5	Yes[14, 15]	1
GNRHR p.P146S		4/5		76
GNRHR p.R139H	LOF[13, 16]	5/5	Yes[15, 16]	13
GNRHR p.A129D	LOF[11, 13, 17]	5/5	Yes[17]	1
GNRHR p.A127V		3/5		9
GNRHR p.Y126C		4/5		1
GNRHR p.P96S		4/5	Yes[15, 18]	1
GNRHR p.V94A		4/5		15
GNRHR p.T91P		4/5		1
GNRHR p.S74X	nonsense	—		1
GNRHR p.W63C		4/4		3
GNRHR p.F46S		4/5		1
HS6ST1 p.G396C		3/4		1
HS6ST1 p.R382W	Yes[19]	4/4	Yes[19]	34
HS6ST1 p.R378P		3/4		2
HS6ST1 p.R378C		3/4		2
HS6ST1 p.R375C		4/4		1
HS6ST1 p.R371C		4/4		3
HS6ST1 p.R367H		3/4		1
HS6ST1 p.R367C		4/4		1
HS6ST1 p.D342G		3/4		1
HS6ST1 p.R336C		4/4		1
HS6ST1 p.R335W		4/4		4
HS6ST1 p.V327M		3/4		1
HS6ST1 p.R323Q		3/4	Yes[19]	1
HS6ST1 p.R323W		4/4		1
HS6ST1 p.T322M		3/4		1
HS6ST1 p.R314Q		3/4		5
HS6ST1 p.T307M		4/4		1
HS6ST1 p.R306W	Yes[19]	4/4	Yes[19]	2
HS6ST1 p.R214H		3/4		2
HS6ST1 p.S207L		3/4		1
HS6ST1 p.R196Q		3/4		1
HS6ST1 p.R190C		4/4		1
HS6ST1 p.G39E		3/3		1
IL17RD p.A735V		3/4	Yes[1]	9
IL17RD p.A722G		3/4		1
IL17RD p.A722T		3/4		1
IL17RD p.L715P		4/4		1
IL17RD p.S712C		4/4		1
IL17RD p.G701D		3/4		1
IL17RD p.E673Q		4/5		1
IL17RD p.S671L		4/4		4
IL17RD p.S667L		3/4		2
IL17RD p.Y665C		4/4		2
IL17RD p.S662X	nonsense	—		1

IL17RD p.P659L		4/4		1
IL17RD p.H648P		3/4		1
IL17RD p.G631W		3/4		1
IL17RD p.Q563X	nonsense	—		1
IL17RD p.E561K		4/4		1
IL17RD p.P557R		4/4		4
IL17RD p.Q551X	nonsense	—		1
IL17RD p.M549R		4/4		8
IL17RD p.I546T		4/5		2
IL17RD p.S541F		4/4		2
IL17RD p.R540W		3/4		10
IL17RD p.S536R		4/4		2
IL17RD p.S536G		4/5		4
IL17RD p.R514X	nonsense	—		1
IL17RD p.S513F		4/4		5
IL17RD p.S513C		4/4		1
IL17RD p.P505L		4/4		2
IL17RD p.D493Y		4/4		2
IL17RD p.P489S		4/4		1
IL17RD p.Y479X	nonsense	—		1
IL17RD p.I457M		4/5		4
IL17RD p.S444W		3/4		1
IL17RD p.V421A		4/5		8
IL17RD p.I419T		4/5		7
IL17RD p.W407S		4/4		1
IL17RD p.D392V		4/4		2
IL17RD p.L381F		4/5		1
IL17RD p.Y379C		3/4	Yes[1]	9
IL17RD p.Y343C		3/4		1
IL17RD p.R322C		4/4		6
IL17RD p.T315M		3/4		6
IL17RD p.A314V		3/4		1
IL17RD p.P293Q		4/4		1
IL17RD p.R244X	nonsense	—		2
IL17RD p.F242S		4/4		1
IL17RD p.E239K		4/5		1
IL17RD p.K235R		4/5		1
IL17RD p.P222L		4/4		2
IL17RD g.64198G>A	splice-site	—		1
IL17RD p.P191L		4/4		3
IL17RD g.64151A>G	splice-site	—		1
IL17RD p.R183Q		3/4		2
IL17RD p.E172K		4/5		1
IL17RD p.P165S		4/4		1
IL17RD p.V161I		4/5		2
IL17RD p.T157M		4/4		2
IL17RD g. 60115G>A	splice-site	—		1
IL17RD p.P133L		3/4		5
IL17RD p.D132E		4/5		5
IL17RD p.K131T	LOF[1]	3/5	Yes[1]	58
IL17RD p.Q126X	nonsense	—		1
IL17RD p.S120L		3/4		2

IL17RD p.L118P		3/4		1
IL17RD p.L115P		3/4		1
IL17RD p.P100L		3/4		1
IL17RD p.V92G		4/4		4
IL17RD p.I75M		4/5		4
IL17RD c.212_213del: p.G71fs	frameshift	—		1
IL17RD c.206dupC: p.P69fs	frameshift	—		1
IL17RD p.Y66C		4/4		2
IL17RD p.T38I		3/4		1
KAL1 g.197870T>A	splice-site	—		3
KAL1 p.P653L		4/5		8
KAL1 p.T649M		3/5	Yes[5, 6]	3
KAL1 p.P644L		5/5		4
KAL1 p.G641R		5/5		3
KAL1 g.196597G>A	splice-site	—		2
KAL1 p.L612V		3/5		5
KAL1 p.Q608L		5/5		4
KAL1 p.N599K		4/5		6
KAL1 p.A590V		3/5		1
KAL1 p.F569L		3/5		1
KAL1 p.G567S		4/5		6
KAL1 p.N564S		3/5		2
KAL1 p.S555C		5/5		3
KAL1 p.G528W		4/5		3
KAL1 p.P521S		5/5		2
KAL1 p.P521T		4/5		1
KAL1 p.R506W		4/5		1
KAL1 p.R461W		4/5		2
KAL1 p.G432R		4/5		2
KAL1 p.R427C		5/5		4
KAL1 p.S396L		3/5	Yes[20]	19
KAL1 p.R384W		5/5		2
KAL1 p.T378M		3/5		1
KAL1 p.Q375R		3/5		1
KAL1 p.G354W		5/5		1
KAL1 p.R348W		3/4		2
KAL1 p.V330A		3/5		2
KAL1 p.R282H		3/5		1
KAL1 p.R282C		5/5		2
KAL1 p.R282S		3/5		3
KAL1 p.A241T		3/5		1
KAL1 p.S230R		3/5		2
KAL1 p.K185N		4/5	Yes[5, 21]	32
KAL1 p.H170Q		3/5		5
KAL1 p.A144P		4/5		2
KAL1 p.D133N		3/5		3
KISS1 p.R120S		4/4		1
KISS1 p.W114C		4/4		1
KISS1 p.N113K		4/4		1
KISS1 p.P95R		4/4		1

KISS1 p.S77I		3/4	Yes[22]	1
KISS1 p.G35S		3/3	Yes[22]	1
KISS1R p.Y65C		4/5		1
KISS1R p.R74G		3/5		3
KISS1R p.N78S		3/5		1
KISS1R g.2203G>T	splice-site	—		1
KISS1R p.T132I		4/5		13
KISS1R p.M136R		4/5		1
KISS1R p.A159E		4/5		1
KISS1R p.S170C		4/5		1
KISS1R p.S197I		4/5		4
KISS1R p.A203D		5/5		1
KISS1R p.W276C		5/5		1
KISS1R p.P278S		4/5		1
KISS1R p.L283P		3/5		1
KISS1R p.Q286X	nonsense	—		1
KISS1R p.Y302H		3/5		5
KISS1R p.Y313H	LOF[23]	4/5	Yes[23]	2
KISS1R p.L321P		5/5		1
KISS1R p.A324T		3/5		1
KISS1R p.S328X	nonsense	—		1
KISS1R p.R331Q		3/5		1
KISS1R p.F334L		3/5		1
KISS1R c.1005delC: p.R335fs	frameshift	—		1
KISS1R p.C389X	nonsense	—	Yes[5]	7
NELF p.D528Y		4/4		1
NELF p.D528N		3/4		2
NELF p.R518H		4/4		1
NELF p.R513C		4/4		1
NELF p.T505M		3/4		1
NELF g. 9842A>C	splice-site	—		1
NELF p.G499R		3/3		1
NELF p.L495H		4/4		1
NELF p.Y485C		3/4		1
NELF p.N477S		3/4		1
NELF p.R446C		4/4		1
NELF p.R443W		4/4		1
NELF p.H439Y		3/4		2
NELF p.M437V		3/4		1
NELF g. 9842A>C	splice-site	—		1
NELF p.I391T		4/4		1
NELF p.P370L		4/4		1
NELF p.R367Q		4/4		1
NELF p.R367W		4/4		2
NELF p.R366H		4/4		1
NELF p.I364V		4/5		1
NELF c.1146_1147insG: p.P5fs	frameshift	—		4
NELF p.G342V		4/4		10
NELF p.T340I				2

NELF p.A335T		3/4		1
NELF p.L325R		4/4		1
NELF p.E323K		4/4		2
NELF p.T317M		4/4		2
NELF p.S314C		4/4		1
NELF g.6153A>G	splice-site	—		1
NELF p.S305C		4/4		3
NELF p.R283H		4/4		1
NELF p.E251Q		4/5		1
NELF p.R250Q		4/4		3
NELF p.R250W		4/4		1
NELF p.R247H		4/4		1
NELF p.E244D		4/5		1
NELF p.A243T		4/5		3
NELF g. 2925G>A	splice-site	—		1
NELF p.R215H		4/4		2
NELF p.R215C		4/4		3
NELF p.P213L		3/4		7
NELF p.D211Y		4/4		1
NELF p.R196C		4/4		1
NELF p.S193C		3/4		2
NELF p.R180W		3/4		3
NELF p.Q172H		4/5		1
NELF p.R161H		3/3		4
NELF p.R161C		3/3		2
PROK2 p.R117W		5/5		1
PROK2 p.C115Y		5/5		1
PROK2 p.R101W		5/5	Yes[2]	9
PROK2 c.296dupT:p.F99fs	frameshift	—		8
PROK2 p.R73H		3/5	Yes[5]	1
PROK2 p.R73C		5/5	Yes[20, 24]	4
PROK2 p.S67R		4/5		4
PROK2 p.G62D		5/5		2
PROK2 p.I55X	nonsense	—	Yes (Unpublished)	13
PROK2 p.K53N		3/5		1
PROK2 p.S49N		4/5		1
PROK2 p.G41D		5/5	Yes[25]	22
PROK2 p.Q39R		4/5		5
PROKR2 p.T335M		3/5		1
PROKR2 c.990delC: p.T330fs	frameshift	—		1
PROKR2 p.E319K		3/5		2
PROKR2 p.Y311C		4/5		1
PROKR2 p.H310Q		3/5		1
PROKR2 p.D299V		3/5	Yes[26]	1
PROKR2 p.G293S		3/5		3
PROKR2 p.P290S	LOF[27]	4/5	Yes[20]	9
PROKR2 p.A289E		5/5		1
PROKR2 p.W288R		5/5		1
PROKR2 p.C287G		4/5		1

PROKR2 p.T282M		3/5		1
PROKR2 p.V274D	LOF[28]	4/5	Yes[29]	1
PROKR2 p.T273M		3/5		3
PROKR2 p.R270H		4/5		4
PROKR2 p.R270C		4/5	Yes[21]	1
PROKR2 p.R268H		5/5		18
PROKR2 p.R264C		4/5		1
PROKR2 p.T260M	LOF[28]	4/5	Yes[21]	5
PROKR2 p.G257E		4/4		9
PROKR2 p.R248W		3/5		1
PROKR2 p.G234D		4/5	Yes[30]	2
PROKR2 p.V233M		3/5		1
PROKR2 p.L218P		3/5		2
PROKR2 p.R164P		4/5		3
PROKR2 p.K162X	nonsense	—	Yes[6]	1
PROKR2 p.A148T		3/5		1
PROKR2 p.R135H		4/5		1
PROKR2 p.R135C		4/5		6
PROKR2 p.R117W		4/5		2
PROKR2 p.V115M	LOF[31]	4/5	Yes[31]	1
PROKR2 p.D112Y		4/5	Yes[6]	1
PROKR2 p.D99N		3/5		1
PROKR2 p.S98Y		4/5		1
PROKR2 p.A96T		3/5		1
PROKR2 p.N86D		3/5		1
PROKR2 p.R85C	LOF[27, 31, 32]	4/5	Yes[31]	39
PROKR2 p.R80S		3/5		1
PROKR2 p.G70S		4/5		3
PROKR2 p.G68S		4/5		2
PROKR2 p.G57V		3/5		1
PROKR2 p.G57C		3/5		1
PROKR2 c.58delC: p.H20fs	frameshift	—		8
SEMA3A p.R734W		4/5		1
SEMA3A p.T717I		3/5	Yes (Unpublished)	106
SEMA3A p.P713A		4/5		1
SEMA3A p.A651V		5/5		1
SEMA3A p.A651T		3/5		1
SEMA3A p.R637H		4/5		4
SEMA3A p.G633V		5/5		1
SEMA3A p.E620K		4/5		3
SEMA3A p.R613X	nonsense	—		1
SEMA3A p.A604P		5/5		1
SEMA3A p.S601L		4/5		1
SEMA3A p.K600M		5/5		5
SEMA3A p.E589Q		3/5		2
SEMA3A p.L564R		4/5		1
SEMA3A p.R554G		5/5		1
SEMA3A p.R552C		5/5		2
SEMA3A p.R544C		4/5		1
SEMA3A p.R531Q		5/5		1

SEMA3A p.R531X	nonsense	—		1
SEMA3A p.A530S		3/5		1
SEMA3A p.L529V		4/5		1
SEMA3A p.R516Q		3/5		2
SEMA3A p.R516W		5/5		6
SEMA3A p.Q511H		3/5		1
SEMA3A p.G508E		5/5		1
SEMA3A p.P486L		3/5	Yes (Unpublished)	2
SEMA3A p.P486Q		3/5		1
SEMA3A p.R484W		5/5		12
SEMA3A p.W469C		4/5		1
SEMA3A p.D454V		4/5		1
SEMA3A p.F450L		4/5		1
SEMA3A p.D447G		4/5		4
SEMA3A p.R438Q		3/5		1
SEMA3A p.V436I		3/5		2
SEMA3A p.P407L		4/5		1
SEMA3A p.I400T		4/5		1
SEMA3A p.P382A		3/5		1
SEMA3A p.R359S		4/5		1
SEMA3A p.R359W		5/5		1
SEMA3A p.G354S		4/5		1
SEMA3A p.S331C		4/5		1
SEMA3A p.H304Q		4/5		1
SEMA3A p.T303A		4/5		1
SEMA3A p.R290H		5/5		1
SEMA3A p.K282R		3/5		1
SEMA3A p.R264T		5/5		2
SEMA3A p.R248H		4/5		8
SEMA3A p.R248C		5/5		2
SEMA3A p.E214G		4/5		1
SEMA3A p.G189A		3/5		1
SEMA3A p.S188C		5/5		1
SEMA3A g.432261G>A	splice-site	—		1
SEMA3A p.E163V		4/5		1
SEMA3A p.A106S		3/5		1
SEMA3A p.R66W	LOF[33]	4/4	Yes[33]	55
SEMA3A p.Y36C		4/4		1
SPRY4 c.896dupT: p.F299fs	frameshift	—		1
SPRY4 p.R274H		5/5		1
SPRY4 p.R274C		5/5		2
SPRY4 p.P271S		3/5		1
SPRY4 p.R267C		3/5		1
SPRY4 p.R263C		4/5		1
SPRY4 p.V244E		4/5		1
SPRY4 p.V244M		3/5		2
SPRY4 p.R234H		5/5		1
SPRY4 p.S229F		4/5		2
SPRY4 p.C226Y		5/5		1

SPRY4 p.T198M		5/5		1
SPRY4 p.C182G		5/5		9
SPRY4 p.S178F		5/5		1
SPRY4 p.R174Q		3/5		3
SPRY4 p.R174W		4/5		3
SPRY4 p.E160K		3/5		5
SPRY4 p.G145C		4/4		1
SPRY4 p.R130H		3/4		1
SPRY4 p.R130C		3/4		1
SPRY4 p.P117L		3/4		1
SPRY4 p.G94R		3/4		2
SPRY4 p.R80C		4/4		4
SPRY4 p.R68W		3/4		6
SPRY4 p.R66W		3/4		7
SPRY4 p.R30Q		4/4		1
SPRY4 p.M17T		3/4		1
SPRY4 c.12delG: p.P4fs	frameshift	—		1
TAC3 g.16064T>C	splice-site	—		1
TAC3 p.V87E		5/5		1
TAC3 p.F85I		4/5		1
TAC3 p.H83R		5/5		14
TAC3 p.P77S		3/5		1
TAC3 p.S65N		4/5		1
TAC3 p.A9G		3/5		1
TACR3 p.R425W		3/5		1
TACR3 p.Y382C		3/5		3
TACR3 p.C374W		5/5		1
TACR3 p.R372H		4/5		1
TACR3 p.R364Q		5/5	Yes[25]	4
TACR3 p.R364X	nonsense	—		1
TACR3 p.P353S	LOF[34]	5/5	Yes[34]	1
TACR3 p.T349P		4/5		1
TACR3 p.W343X	nonsense	—		1
TACR3 p.I320V		3/5		1
TACR3 p.V298I		3/5		3
TACR3 p.W275C		5/5		1
TACR3 p.W275X	nonsense	—	Yes[25, 35, 36]	25
TACR3 p.T266I		3/5		1
TACR3 p.V255M		3/5		1
TACR3 p.R230H		4/5		3
TACR3 p.L213P		4/5		3
TACR3 p.P190A		4/5		4
TACR3 p.A180E		5/5		1
TACR3 p.T177M		3/5		1
TACR3 p.S172T		4/5		1
TACR3 p.A146V		3/5		1
TACR3 p.A146T		3/5		1
TACR3 p.D131N		3/5		1
TACR3 p.Y122H		4/5		1
TACR3 p.N121S		4/5		1
TACR3 p.Y92C		5/5		1

TACR3 p.F78L		3/5	1
TACR3 p.W41X	nonsense	—	1
WDR11 p.T5K		3/4	1
WDR11 p.V6M		4/5	1
WDR11 p.R13C		4/5	1
WDR11 p.R85W		4/4	1
WDR11 p.L132V		4/5	5
WDR11 p.N145I		4/5	2
WDR11 p.T150I		4/5	2
WDR11 p.S165C		3/4	2
WDR11 p.S186P		3/4	1
WDR11 p.S208C		3/4	10
WDR11 p.Q249X	nonsense	—	1
WDR11 p.R266P		4/5	1
WDR11 c.810delC:p.I270fs	frameshift	—	3
WDR11 p.L273P		4/5	3
WDR11 p.R286C		3/4	3
WDR11 p.Q293X	nonsense	—	1
WDR11 g.14447G>A	splice-site	—	1
WDR11 g.14447_14448insC	splice-site	—	1
WDR11 p.C297R		3/4	1
WDR11 p.C305Y		4/4	1
WDR11 p.V380F		3/4	3
WDR11 p.W383R		4/4	2
WDR11 p.C516W		3/4	1
WDR11 p.S531C		4/5	3
WDR11 p.A533V		4/5	1
WDR11 p.P591S		3/4	1
WDR11 p.R598W		4/4	1
WDR11 p.R640C		3/4	1
WDR11 p.R703W		4/4	1
WDR11 p.R703Q		3/4	1
WDR11 p.L723F		4/5	1
WDR11 p.S742Y		3/4	1
WDR11 p.H748R		4/4	1
WDR11 p.L766S		4/5	1
WDR11 p.Y770C		3/4	1
WDR11 p.R792I		3/4	1
WDR11 p.R797C		4/4	1
WDR11 p.C804R		3/4	5
WDR11 p.C804Y		4/4	1
WDR11 g.39707T>C	splice-site	—	1
WDR11 p.L847V		4/5	1
WDR11 p.R918W		4/4	1
WDR11 p.Y920C		3/4	4
WDR11 p.S924L		4/4	2
WDR11 p.Y935C		3/4	1
WDR11 p.C966R		4/4	2
WDR11 c.3006delC: p.D1002fs	frameshift	—	1

WDR11 p.G1008D		3/4	6
WDR11 p.S1042L		4/4	1
WDR11 p.Q1047X	nonsense	—	1
WDR11 g.54135A>G	splice-site	—	1
WDR11 g.54136G>A	splice-site	—	2
WDR11 p.D1078N		4/5	1
WDR11 p.T1085I		4/4	1
WDR11 p.R1091W		4/4	2
WDR11 p.W1094R		3/4	1
WDR11 p.D1107N		4/5	2
WDR11 p.R1111W		4/4	1
WDR11 p.L1116F		4/5	1
WDR11 p.S1133P		4/4	1
WDR11 p.G1135D		4/4	2
WDR11 p.L1144H		4/5	1
WDR11 p.A1154T		4/5	1
WDR11 p.F1156S		3/4	1
WDR11 p.K1162T		3/4	1
WDR11 p.G1191S		4/5	13
WDR11 p.G1204R		3/4	2
WDR11 p.L1211S		4/5	4

Abbreviation: ExAC, Exome Aggregation Consortium; LOF, loss-of-function as indicated by significantly decreased performance in *in vitro* functional assays compared to wild-type; Ref., reference; —, not tested

*Not all prediction programs gave predictions for all variants.

†Each allele with a potentially pathogenic variant was counted as one individual subject for statistical analysis.

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